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The Journal of Biochemistry, Vol. 47, No. 4, 1960

STUDIES ON ENZYMATIC SYNTHESIS OF COCARBOXYLASE IN ANIMAL TISSUE

IV. EFFECT OF METALLIC IONS, VARIOUS NUCLEOTIDES AND THIAMINE DERIVATIVES ON THIAMINOKINASE FROM RAT LIVER

By YOSHITAKE MANO AND RYO TANAKA

(From the Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo)

(Received for publication, September 8, 1959)

In the earlier communications of this series (*1*, *2*, *3*), some properties of thiaminokinase from rat liver were described. Activity of this enzyme was influenced diversely by various internal and external factors, and various compounds besides essential factors markedly affected the extent of this reaction. These factors complicate the pattern of the specificity so that its whole aspect cannot be expressed in brief.

The present paper deals with the effect of metallic ions and the apparent specificity of substrates, phosphate donor and acceptor, and refers also to the effect of the related compounds on the enzyme activity.

METHODS AND MATERIALS

The enzyme used in the experiments was prepared according to the description of the previous paper (*3*), where the purity of the preparation was indicated in each step of the purification. Unless otherwise specified, the enzyme activity was determined under the standard conditions described in the first communication (*1*). In the experiments on the effect of various nucleoside triphosphates, $5 \times 10^{-4}\,M$ of neopyrithiamine hydrobromide was added to the apocarboxylase system to prevent the activity of thiaminokinase contaminated in the preparation, because neopyrithiamine was found to be a specific inhibitor of yeast thiaminokinase (*4*). The assay for the activity of nucleoside diphosphokinase was carried out by the method of Kurahashi *et al.* (*5*) using crystalline pyruvate kinase and crystalline lactic dehydrogenase, or by the method of Kaziro (*6*) using yeast thiaminokinase at appropriate pH.

Nucleoside mono-, di- and triphosphates were obtained from the Pabst Laboratories and the Sigma Chemical Company, neopyrithiamine was purchased from the Sigma Chemical Company, and other thiamine derivatives were kindly supplied by Dr. Matsukawa of the Takeda Pharmaceutical Industries, Ltd. The purity of all these substrates was checked by paper chromatography or by spectrophotometric measure-

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The following abbreviations are used in this paper: 5′-phosphate, diphosphate, and triphosphate of adenosine, guanosine, inosine, uridine and cytidine, AMP, ADP, ATP, GMP, GDP, GTP, IMP, IDP, ITP, UMP, UDP, UTP, CMP, CDP and CTP; thiamine-pyrophosphate, TDP; tris (hydroxymethyl) aminomethane, Tris.

ment, and for possible impurities, if present, ion exchange column chromatographic separation was applied on the specified compounds.

RESULTS

Effect of Metallic Ions—As has been reported in the earlier paper (*1*), the reaction requires essentially divalent cations. Table I shows that divalent cations such as Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} are effective as essential factor, and some of other cations have less but distinct effect. Each of the above

TABLE I

Effect of Various Cations

Each vessel contained : 10^{-4} *M* thiamine, 10^{-3} *M* metallic ions, 10^{-3} *M* ATP, 2.8×10^{-2} *M* Tris buffer of pH 8.0 and enzyme derived from Step 5 (*3*). Total volume, 3.0 ml. Incubation, 37° for 60 minutes.

TDP formed was determined by the same way as described in Paper I (*1*).

Cation added	Rate of activity to that of Mg^{++}-activated system of the same concentration (10^{-3} *M*)	Effect on Mg^{++}-activated system (percentage to the control system)
None	0	100
Ba^{++}	12	115
Sr^{++}	38	118
Ca^{++}	30	44
Mg^{++}	100	—
Al^{+++}	12	112
Mn^{++}	32 (158c))	21
Fe^{++}	25	81
Fe^{+++}	4	96
Co^{++}	87	74
Ni^{++}	84	72
Zn^{++}	11	22
Sn^{++}	3	103
Pb^{++}	9	109
Cd^{++}	0	74d)
Cu^{++}a)	0	78d)
Hg^{++}	0	84d)
Ag^{+}b)	0	81d)

All the cations were used as chloride except a) and b), which were used as sulfate and nitrate, respectively. c) 3.3×10^{-4} *M* cations.

d) Value calculated according to the formula, $x\cdot\frac{100}{100\text{-}a}$ where x is the percentage of the effect on the Mg^{++}-activated system, obtained by the determination of TDP carried out at 5×10^{-5} *M* of each of the metallic ions, and a is percentage inhibition of these metallic ions to the carboxylase system where the concentration of TDP was 150 mμg.

essential ions had a definite optimal concentration which was related closely to the concentration of ATP, without any noticeable relation with the concentration of other components such as thiamine and enzyme. The optimal ratio of the concentrations of ATP/metal was about 3 in the case of Mn^{++} and about 1 in the cases of Co^{++} and Ni^{++}, whereas Mg^{++} showed no distinct but a broad optimum under the conditions tested (Fig. 1). Another marked difference of these metallic ions was in their effect on the relation of nucleotides used, pH and activity, which will be described in detail under the section on the "Effect of nucleotides".

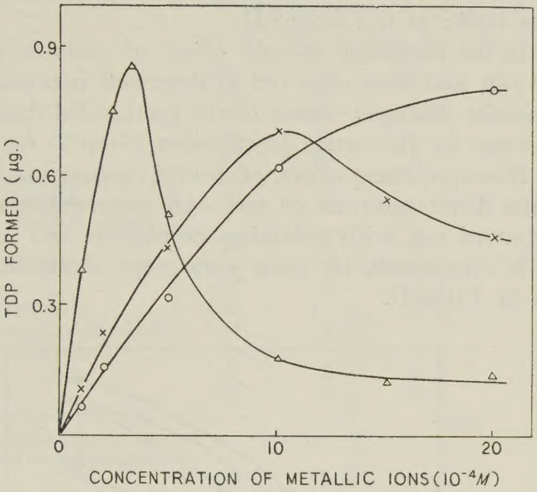

FIG. 1. Effect of concentration of various cations.

Each vessel contained : 10^{-4} *M* thiamine, 10^{-3} *M* ATP, enzyme from Step 4, divalent cations (chloride) as indicated, and 2.8×10^{-2} *M* Tris buffer of pH 8.0 for the Mn^{++}- and Mg^{++}-system and 3.3×10^{-2} *M* acetate buffer of pH 5.5 for the Co^{++}-system. Total volume, 1.5 ml. Incubation, 37° for 60 minutes. —△—, Mn^{++} ; —○—, Mg^{++}; —×—, Co^{++}.

The effect of various cations on the Mg^{++}-activated system is also shown in Table I. A part of the cations of alkaline earths, Ba^{++} and Sr^{++}, were accelerative, whereas Ca^{++} was inhibitory. The effect of the existence of essential cations, Mg^{++} and Mn^{++}, is worth noting: when Mn^{++} was added to the Mg^{++}-activated system in the same molar concentration, 10^{-3} *M*, activity of the Mg^{++}-activated system declined remarkably to fall into the normal value of the Mn^{++}-activated system, and this feature was independent on order of the addition of Mn^{++} and Mg^{++}. Other essential cations, Co^{++} and Ni^{++}, when added to the Mg^{++}-activated system, showed some inhibition, but less effective cations like Fe^{++} showed smaller extent of inhibition. The effect of heavy metals such as Cu^{++}, Hg^{++} and Ag^{+}, could not be observed because they are strong inhibitor of the apocarboxylase system. Calculated values in these cases showed that they are rather mild inhibitors. Other metals like Zn^{++} showed a remarkable inhibition, but Pb^{++} was characterized by ineffectiveness on the Mg^{++}-activated system. Al^{+++} behaved like Ba^{++} and Sr^{++}. Fe^{+++} and Sn^{++} was neither activator nor inhibitor.

The results shown above were also influenced by the buffer used. For example, the optimal ratio of ATP/metal for Mn^{++} shifted from 3 in the above buffer to about 0.6 in 2.8×10^{-2} *M* Tris-maleate buffer of pH 8.0, and the Ca^{++}-inhibition was reduced from 56 per cent to 22 per cent in 2.5×10^{-2} *M* glycyl-glycine buffer of the same pH.

In addition to the foregoing specific effect of metallic ions, non-specific effect of neutral salt had been observed as described previously (*1*).

Effect of Thiamine Analogues—Some of the synthesized thiamine derivatives serve as a substrate in the crude preparation (Step 2) for the synthesis of cocarboxylase. However, there arises, of course, a question whether each of them is really the direct substrate or not. An experiment on the substrate specificity was carried out with thiamine derivatives and related substances covering about 30 compounds, by using some steps of purification of the enzyme, as shown in Table II.

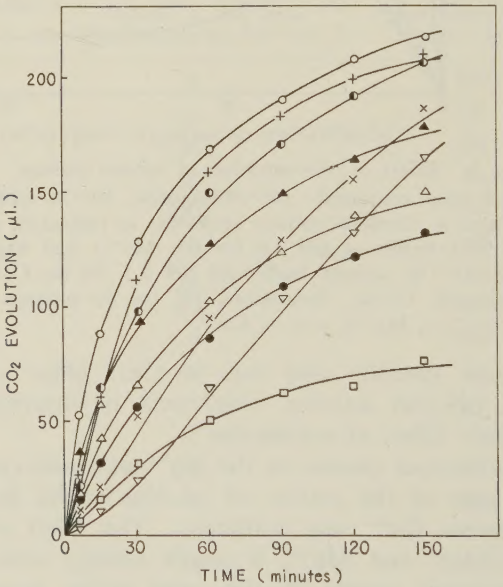

Fig. 2. Time course of the reaction with some effective thiamine derivatives. The reaction condition followed that shown in Table II. The enzyme used was derived from Step 2. —○—, thiamine ; —+—, thiamine disulfide ; —◐—, thiamine allyl disulfide ; —▲—, thiamine propyl disulfide acetyl ester ; —×—, dibenzoylthiamine ; —▽—, diacetylthiamine ; —△—, O-acetylthiamine ; —●—, O-benzoylthiamine ; —□—, ethylthiamine.

With the crude preparation (Step 2) about one-half of the compounds tested were effective as a "substrate". Thiamine, its disulfide compounds,

TABLE II

Effect of Thiamine Analogues on the Reaction

Reaction mixture contained: 10^{-4} M thiamine derivatives, 3.3×10^{-3} M $MgCl_2$, 10^{-3} M ATP, 2.8×10^{-2} M Tris buffer of pH 8.0, and enzyme from specified steps. Total volume, 3.0 ml. Incubation, 37° for 60 minutes.

Additions	Relative activity to that of thiamine as 100			Effect on the thiamine system in relative activity to the control as 100
	Enzyme from			
	Step 2	Step 4	Step 6	Enzyme from Step 4
Thiamine	100	100	100	—
O-Acetylthiamine	70	55	7	106
O-Benzoylthiamine	43	21	6	100
Diacetylthiamine	68	28	3	95
Dibenzoylthiamine	84	18	2	103
O-Benzoyl-S-propionylthiamine	66	11	0	103
Thiamine propyl disulfide	92	8	0	98
Thiamine propyl disulfide acetyl ester	88	2	0	101
Thiamine propyl disulfide propionyl ester	94	6	2	105
Thiamine allyl disulfide	101	12	3	111
Thiamine β-hydroxyethyl disulfide	94	6	0	94
Thiamine tetrahydrofurfuryl disulfide	91	3	0	101
Thiamine disulfide	105	9	0	112
3-(2′-Methyl-4′-aminopyrimidyl)-5′- methylformamino-5-hydroxy-Δ^2 pentenyl (3)-(4″-nitrophenacyl)-thioether	0	0	—	92
3-(2′-Methyl-4′-aminopyrimidyl)-5′-methylformamino-5-hydroxy-Δ^2 pentenyl (3)-(2″-hydroxyethyl)-thioether	2	0	—	97
3-(2′-Methyl-4′-aminopyrimidyl)-5′-methylformamino-5-benzoxy-Δ^2 pentenyl (3)-(2″-hydroxyethyl)-thioether	0	—	—	103
3-(2′-Methyl-4′-aminopyrimidyl)-5′-methylformamino-5-benzoxy-Δ^2 pentenyl (3)-carboxymethyl-thioether	0	—	—	88
Ethylthiamine	27	12	11	62
Butylthiamine	0	0	—	68
Oxythiamine	4	0	—	94
Thiothiamine	2	0	—	102
3-(2′-Methyl-4′-hydroxypyrimidyl)-5′-methyl-4-methyl-5-β-hydroxy-ethylthioazole-2-thione	0	—	—	94
3-(2′-Butyl-4′-aminopyrimidyl)-5′-methyl-4-methyl-5-β-hydroxy-ethylthiazole-2-thione	0	—	—	103
3-(2′-Methyl-4′-aminopyrimidyl)-5′-methyl-4-methyl-5-β-hydroxy-ethylthiazole-2-one	0	—	—	97
1-(2′-Methyl-4′-aminopyrimidyl)-5′-methylpyrimidine	2	0	—	93
Pyrithiamine (neo)	4	0	—	47
Thiochrome	0	—	—	102
2-Methyl-4-amino-5-hydroxy-methyl-pyrimidine	—	—	—	104
4-Methyl-5-β-hydroxyethylthiazole	—	—	—	98

its ester derivatives, and ethylthiamine were effective as a "substrate", whereas S-alkyl derivatives, thiothiamine derivatives, the substituents of thiazole moiety and 6-amino group of pyrimidine moiety, were all inactive. By testing the time course of cocarboxylase-formation on these effective compounds, the mode of formation would be differentiated into four types as shown in Fig. 2. These are as follows:

Type I: The curves resemble that of thiamine and have no lag phase. The values do not exceed thiamine. This type contains simple disulfide compounds.

Type II: This group has a steady and rather slow formation of cocarboxylase at the initial stage without distinct lag phase, followed by relatively early decline of the rate. This contains the compounds having monoester linkage in their structure.

Type III: This group has a marked lag phase in the curve at the initial reaction phase, which suggests a participation of some other enzyme for the appearance of the activity. Diester derivatives of thiamine belong to this group.

Type IV: This group shows a curve similar to that of Type II, but the level is the lowest among the active substrates. This type includes ethylthiamine only.

With the progress of purification of the enzyme the activity pattern of thiamine derivatives altered at each step (Table II); namely, at the step of ammonium sulfate fractionation every disulfide derivative lost its activity, then at the step of acid treatment the activity of the diester compounds disappeared, and the only remaining monoester group of the alcoholic residue lost their activity at the step of Cγ-gel fractionation. Although a considerable part of the activity of ethylthiamine was lost at Step 4, low but constant activity remained in the compound was observed even at Step 6. None of other enzyme fractions discarded was found to be active with any of these derivatives.

These facts indicate that the direct acceptor for the synthesis of cocarboxylase is thiamine as far as were tested, and others except ethylthiamine would become effective through their secondary conversion to thiamine by the action of specified enzymes. Therefore, the crude preparation would at least contain three different enzymes which convert various thiamine derivatives to thiamine, *i.e.* the enzymes for splitting the disulfide bond, the thioester, and the alcoholic ester. It is not known from the experiment whether the remaining slight activity of ethylthiamine originates in its intrinsic nature or not. The presence of an enzyme which splits alkyl chain remains in question. The above experiment represents by no means the substrate specificity of thiamine derivatives as the phosphate acceptor, because the determination system, as far as is known at present, is only specific for TDP.

The effect of addition of these compounds to the thiamine system is also indicated in Table II. Although almost all of the compounds tested had no serious effect on the system, there can be noted several exceptions; *viz.*

ethylthiamine, butylthiamine and neopyrithiamine act as inhibitor. The mechanism of inhibition of these compounds were disclosed to be of competitive nature from their Lineweaver-Burk's plots (*7*), and their *Ki* were estimated from Dixon's plots (*8*) to be 4.1×10^{-5}, 4.3×10^{-5} and 3.3×10^{-5} *M*, respectively, for the above three inhibitors. These three inhibitors had no specific action in reaction between cocarboxylase and apocarboxylase, although ethylthiamine showed moderate Ochoa's effect (*9*) in the case with crude apocarboxylase preparation.

Effect of Nucleotides—Various nucleoside phosphates were compared as to their effectiveness as a pyrophosphate donor using various steps of purification of the enzyme. As shown in Table III, with the original extract (Step 2) ATP and UTP were effective as phosphate donor, ITP and GTP were effective to a lesser extent, and CTP had practically no activity. Among nucleoside diphosphates and monophosphates, ADP was effective using the crude preparation as has been pointed out previously (*1*), in contrast to the inactivity of GDP, IDP, UDP, CDP, AMP, GMP, and CMP, and the activity of ADP was lost with the preparation of the advanced step (Step 6). With the purest enzyme (Step 6), only ATP and UTP remained as the substrate, and the ratio of activity of ATP to UTP somewhat increased with the purification steps (Table III).

TABLE III

Comparative Effect of Various Nucleoside Triphosphates

Each vessel contained : 10^{-4} *M* thiamine, 3.3×10^{-3} *M* $MgCl_2$, 10^{-3} *M* nucleoside triphosphate, 2.8×10^{-2} *M* Tris buffer of pH 8.0 and the enzyme. Total volume, 1.5 ml. and the mixture was incubated for 60 minutes at 37°. Values are expressed in relative activity to that of ATP as 100 in each enzyme preparation.

Enzyme	ATP	GTP	ITP	UTP	CTP
Step 2	100	19	24	66	7
Step 4	100	3	5	36	0
Step 6	100	0	2	41	1

In the crude extract (Step 2) a slight activity of nucleoside diphosphokinase could be detected, which could not, however, be demonstrated with the purest preparation (Step 6). Deprivation of possible phosphate acceptor of nucleoside diphosphokinase from the crude preparation (Step 2) by means of treatment with Dowex 1, Norit A at a low pH, dialysis, or electrodialysis resulted in a loss of activity in the case of ITP and GTP, while ATP and UTP still remained active. Addition of ITP and GTP to UDP with the dialyzed crude extract caused slight recovery of the activity, but addition of CTP was not the case. These findings suggest that the activity of ITP and GTP might not be due to their inherent nature but resulted through a secondary nature, after conversion to the "natural substrate", ATP and UTP, by the action of nucleoside diphosphokinase coupling with nucleoside diphosphates contaminated in the crude enzyme preparation. Difference of the activity ratio of ATP to UTP between the two preparations from Step 2 and 4 may in part be caused by the same reason.

Crude enzyme preparations (Step 2 and 3) also contained an enzyme which degrades ITP strongly and fairly specifically, but the enzyme did not degrade GTP so strongly. As the decomposition of ITP, GTP, and CTP might be the possible cause of their inactivity, it was checked over each step of the purification. The results revealed that the inactivity of these nucleotides was not caused by their decomposition because no noticeable activity was observed even under the conditions which kept these nucleotides surviving. Slight activity which was observed in the alkaline side in the cases of ITP, GTP, and CTP might not be attributed to specific character because no distinct relation between the activity and their concentrations was observed. Therefore, it can be concluded that ITP, GTP, and CTP do not act as a true donor under the conditions studied. All efforts to find possible fraction effective to these nucleotides in the discarded part of the extract and for possible conditions for their effectiveness ended in failure.

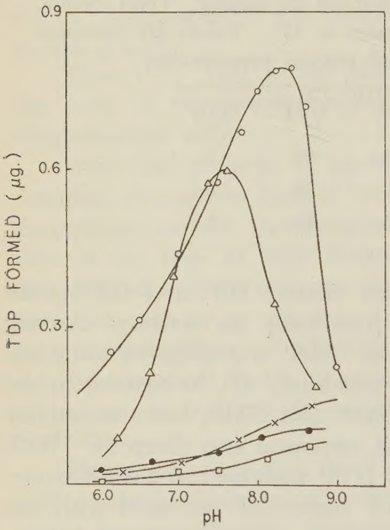

FIG. 3. pH-activity curves with various nucleoside triphosphates.

Reaction conditions: A mixture (1.5 ml.) of 10^{-4} M thiamine, 3.3×10^{-4} M $MnCl_2$, 10^{-3} M nucleoside triphosphate, 2.8×10^{-2} M Tris-maleate buffer and enzyme from Step 6 was incubated at 37° for 60 minutes. —○—, ATP; —△—, UTP; —×—, ITP; —●—, GTP; —□—, CTP.

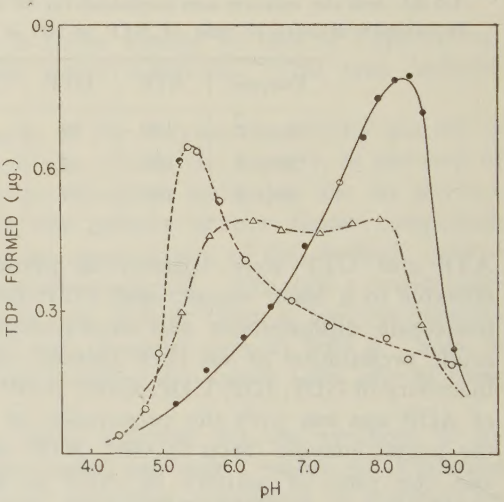

FIG. 4. pH-activity curves with different cations.

Reaction mixture consisted of: 10^{-4} M thiamine, 10^{-3} M ATP, divalent cations (10^{-3} M $MgCl_2$, $CoCl_2$ or 3.3×10^{-4} M $MnCl_2$ for each specified system), buffer as indicated, and enzyme derived from Step 6. Total volume, 1.5 ml. Incubation, 37° for 60 minutes. —●—, Mn^{++}; —·—△—·—, Mg^{++}; ---○---, Co^{++}.

Comparison of the activity of ATP and UTP showed a remarkable variety under reaction condition besides the enzyme purity mentioned above. The most striking effects were those of pH and metallic ions, and both effects were closely related to each other as shown in Figs. 3 and 4. In the case of Mn^{++}, ATP and UTP had different pH optima, at pH 8.3–8.4 and 7.5–7.6, respectively, but in the case of Mg^{++}, both nucleotides had practically similar broad optima covering pH 6 to 8 though the two curves showed some discrepancy. When Co^{++} and Ni^{++} were employed approximately the same optima at pH 5.4–5.5 were observed on both nucleotides. Furthermore, the effect of these ions may also vary with purity of the enzyme. The effect of Mg^{++} showed no great difference among the preparations derived from each step of the purification, while in the case of Mn^{++} majority of the effect found in the crude enzyme disappeared in purer preparations obtained after Step 4 in spite of the choice of the optimal condition.

On use of these metallic ions the optimal ratio of concentrations between nucleotides and metallic ions varied with the nucleotides used, and the optimal ratio of UTP ($2\times10^{-4}\,M$) to Mn^{++} at pH 7.5 was about 5, while other cations held similar relation as in the case of ATP. The optimal ratios of the concentrations of ATP/metal also changed with pH. For example, at lower pH of 7.2, the characteristic ratios of ATP to Mn^{++} and Co^{++} changed to that of the Mg^{++}-type which was indicated already. From these observations it is conceivable that the activity of some nucleotides, ATP and UTP, varied diversely with the conditions used. As for the question of whether the enzyme corresponding to ATP- and UTP-reaction is a single enzyme or not, no positive evidence was so far obtained which demonstrates that the separate fractions are active to ATP and UTP exclusively.

When various nucleotides were used together, they could interact mutually as was noted in the previous paper on adenine nucleotides (*1*). ITP, GTP, CTP, IDP, GDP, CDP, UDP, IMP, GMP and CMP were also inhibitory to the reaction of ATP, and their inhibition was more extensive than that of AMP and ADP as shown in Table IV. The mechanism of inhibition by IMP was quite different from that of AMP which was considered to be competitive (*1*). Fig. 5 shows inhibition by the addition of IMP to the

TABLE IV

Inhibitory Effect of Various Nucleotides on the ATP System

Each vessel contained: $10^{-4}\,M$ thiamine, $1.65\times10^{-3}\,M$ $MgCl_2$, $5\times10^{-4}\,M$ ATP, $5\times10^{-4}\,M$ nucleotide as indicated, $2.8\times10^{-2}\,M$ Tris buffer of pH 8.0 and enzyme, obtained by the treatment of the preparation from Step 6 with Dowex 1-(Cl^-) at pH 8.0.

Inhibition %											
AMP	IMP	GMP	CMP	ADP	GDP	IDP	UDP	CDP	ITP	GTP	CTP
57	90.6	86.3	87.7	41.6	80.8	85.3	84.7	87.2	84.2	89.0	78.4

ATP-system, and the nature of the inhibition was interpreted to be non-competitive. The values 2.1×10^{-4} and 3.4×10^{-4} *M* were calculated as *Ki* for IMP and GMP, respectively, by the Dixon's plots (*8*).

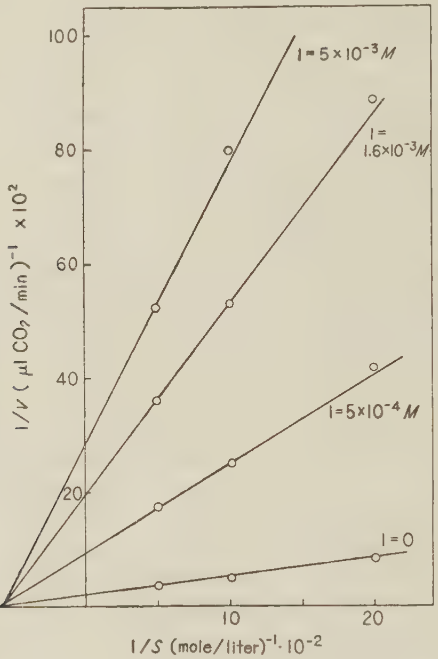

FIG. 5. Lineweaver-Burk's plot of IMP (I) inhibition.

Reaction condition: 10^{-4} *M* thiamine, 3.3×10^{-3} *M* $MgCl_2$, 5×10^{-4} *M* ATP, IMP as indicated, 2.8×10^{-2} *M* Tris buffer of pH 8.0, and tne enzyme derived from Step 5. Incubation at 37° for 15 minutes.

Accordingly, the mode of nucleotides in the reaction is quite intricate, and the whole aspect can be seen only by way of multidimensional observations.

DISCUSSION

The interaction between metallic ions and substrate which affects the enzyme activity has so far been discussed generally by many authors. Considering present experimental evidences, the essential cations, Mg^{++}, Mn^{++} Co^{++} and Ni^{++}, all of which are divalent cations having coordinate number of 6, seem to be closely related to the connection between nucleotides and enzyme forming a sort of metal chelate complex through which the enzyme activity may vary widely. The experimental data shown above seem to suggest that the activity may be influenced by the mode of chelate formation. The fact that the addition of Mn^{++} to the Mg^{++}-activated system decreases the activity to the Mn^{++}-activated state suggests that the dissociation constant between Mn^{++} and enzyme might be smaller than that of Mg^{++} and enzyme. Like other examples (*10*), Ca^{++} or Zn^{++} showed an ionic antagonism against the essential cations. Low sensitivity of the enzyme to heavy metals suggests non-SH nature of the enzyme, which is compatible with unresponsiveness to *p*-chloromercuribenzoate (*1*). The effect of buffer on the action of cations may be caused by the decrease of effective ions through chelation with the buffer.

It has been known that only thiamine (but ethylthiamine) participates as the phosphate acceptor in the biosynthesis of TDP directly. Other thiamine derivatives which are able to form cocarboxylase in the crude preparations may react in an indirect way by the aid of other enzymes leading them to thiamine. The actual existence of such enzymes must be studied further, since the present deduction is based only on the findings on the relation between activity, purification grade of the enzyme, and time course of the reaction. As has been pointed out already, this pattern of substrate specificity cannot represent that of pyrophosphate transfer because determination of the reaction product was carried out only for cocarboxylase activity. Therefore, it cannot be concluded from these experiments whether the production of pyrophosphate esters of these derivatives occurred or not.

The finding that the synthetic ethylthiamine pyrophosphate possesses cocarboxylase activity at about ten per cent of TDP (*11*) suggests the enzymatic formation of the pyrophosphate derivative of ethylthiamine. Ethylthiamine is said to substitute for thiamine *in vivo* (*12*). Nevertheless it has only a small activity as a substrate of thiaminokinase besides acting as a competitive inhibitor. This contradiction yet remains unsolved and other factors might be present *in vivo* system. The problem whether the compounds showing an inhibition are or not converted to the corresponding pyrophosphate esters by the enzyme was not dissolved by the experiment, though it has been reported that only ethylthiamine can be pyrophosphorylated among the inhibitors listed above (*13*, *14*, *15*). It has been observed that ethylthiamine pyrophosphate has binary action of acceleration and inhibition to the carboxylase reaction with cocarboxylase under the conditions tested*. Considering these facts, the mechanism of inhibition caused by ethylthiamine may be more complicated nature in contrast to other inhibitors than such that was indicated in apparent competitive mechanism.

As has been realized from these experiments, response of the reaction to various nucleotides is quite complicated. Alteration of the nucleotide specificity by the condition was recently demonstrated by Kieley *et al.* (*16*) and Kurahashi *et al.* (*6*). Another example of different effects brought about by a particular ion with different substrates is known in the reaction catalyzed by leucine aminopeptidase (*17*). Therefore, the nucleotide specificity may undergo a change with the condition tested, and this phenomenon was also observed partly in the present enzyme.

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* Nakamura, S., unpublished data

Considering together with the results of yeast thiaminokinase (*6*) it would be expected that there might be a suitable condition under which GTP, ITP, and CTP are active, but it is questionable whether the liver enzyme has really an activity to all nucleoside triphosphates under appropriate condition. On the other hand, the difference between the nucleotides specificity of the thiaminokinase from yeast and liver might be mainly due to that of affinity between the enzyme nad the chelate complex of nucleotide and metallic ions.

It has been known that there is a close similarity between GTP and ITP in their behavior in enzyme reactions (*5*, *18*), but little is known about the same relationship between ATP and UTP. Thus an alternative explanation would be that the specific enzyme which require ATP or UTP might exist, and the conclusive evidence against this possibility could not be obtained from the present experiments.

Hitherto reported nucleotide specificity in various enzyme reactions has never been examined under variety of conditions except in the few examples quoted above (*5*, *6*, *16*, *17*). Since the pattern of the specificity cannot be expressed in brief as pointed out already, this problem should be reexamined more carefully in many enzymes under various experimental conditions in general.

SUMMARY

The effects of metallic ions, thiamine derivatives as a phosphate acceptor, and nucleotides as a phosphate donor on rat liver thiaminokinase were investigated and the results were as follows.

1. Metallic ions effective as essential activator were Mg^{++}, Mn^{++}, Co^{++} and Ni^{++} among those tested. These ions had definite molar optimal relations to nucleotides used under the condition tested, but they were variable. Mg^{++} and Mn^{++} were in mutual antagonism to the activity. Ca^{++} and Zn^{++} acted as inhibitor.

2. Various thiamine derivatives except ethylthiamine could not be a substrate for the formation of cocarboxylase by the purified enzyme. Among the derivatives tested, disulfide compounds and ester derivatives of thiamine could serve as a substrate in the crude enzyme, and all of them may be considered to be induced to TDP *via* thiamine. Ethylthiamine was still active in the purest preparation. Ethylthiamine, butylthiamine and neopyrithiamine acted as an inhibitor competitive with thiamine.

3. Nucleotides as pyrophosphate donor were confined to ATP and UTP with the purified enzyme under the condition studied, and other nucleosides triphosphates could act as a substrate only with the crude system presumably by the action of other enzymes deriving them to the effective nucleotides. The activity with the effective nucleotides varied markedly with the reaction condition, such as pH and metallic ion concentrations. Moreover, all the nucleotides tested interacted inhibitorily with ATP upon the activity.

The authors wish to express their sincere thanks to Prof. N. Shimazono for his kind guidance.

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GLUTAMIC ACID FORMATION FROM GLUCOSE BY BACTERIA

III. ON THE PATHWAY OF PYRUVATE FORMATION IN BREVIBACTERIUM FLAVUM NO. 2247

By ISAMU SHIIO, SHIN-ICHIRO ÔTSUKA
AND TOSHINAO TSUNODA

(*From the Central Research Laboratory of Ajinomoto Co., Inc., Kawasaki*)

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In the previous studies, most of the enzymes of the Embden-Meyerhof pathway and glucose-6-phosphate dehydrogenase, one of the enzymes of the hexose monophosphate oxidative cycle, have been demonstrated in cell free extracts of *Brevibacterium flavum*, No. 2247 (*1*). The result suggests the operation of both EM and HMP pathways in this organism for the aerobic oxidation of glucose. Therefore, some tracer experiments using variously labelled glucose would be required for the evaluation of the relative importance of these pathways.

The establishment of the relative contribution of routes of glucose metabolism can be carried out usually by measuring the relative isotopic contribution of various carbons of the glucose molecule to various metabolic products. These methods have been reviewed critically by Wood (*3*). Dawes and Holms (*4*) have observed the conversion of position-labelled glucose substrates to carbon dioxide and pyruvate in the presence of arsenite, and evaluated the role of the two pathways in *Sarcina lutea*.

The preliminary studies revealed that the resting cells of *B. flavum*, which was known to accumulate L-glutamate in the presence of ammonium ions (*2*), aerobically produced a considerable amount of pyruvate from glucose in the presence of arsenite and herewith they absorbed one mole of oxygen for one mole of glucose consumed*.

The present paper deals with the pathway of pyruvate formation from glucose in this organism. The experiments were carried out mostly according to the method described by Dawes and Holms.

The abbreviations used throughout this paper include EM pathway, the Embden-Meyerhof pathway; HMP pathway, the hexosemonophosphate oxidative pathway; ED system, the Entner-Doudoroff system; cpm, counts per minute; DiNPH, 2,4-dinitrophenylhydrazone or -hydrazine.

* unpublished data.

EXPERIMENTALS

Isotopic Materials—Glucose-1- and -6-C^{14} were obtained from the Radiochemical Centre, Amersham, England, and glucose-U-C^{14} from the Institute of Applied Microbiology, University of Tokyo, Tokyo. The radiochemical purity of these substances were 97.6, 98.2, and 82.8 per cent, respectively. The purity was determined as follows: the substance was chromatographed on a paper, the corresponding spot was eluted, and the radioactivity of the eluate was counted (solvent: phenol-water, 10: 2, v/v).

Formation of C^{14}-Pyruvate from C^{14}-Glucose—The experiments on the formation of C^{14}-pyruvate from glucose-1-, -6-, and -U- C^{14} were performed in conventional Warbung vessels under air at 37°. The reaction mixture consisted of 250 μ moles of phosphate, pH 7.0, 125 μ moles of sodium arsenite (freshly neutralized), about 5 μ moles of C^{14}-glucose (giving 2.82, 2.30, and 1.54×10^5 c.p.m for glucose-1-, -6-, and -U- C^{14}, respectively), 0.5 ml. (0.36 mg. N) of washed cell suspension and water in a total volume of 2.5 ml. The center well of vessel contained 0.2 ml. of 20 per cent KOH and a piece of filter paper. After the oxygen absorption ceased (40∼50 minutes), the reaction mixture was centrifuged. Two ml. of 0.4 per cent 2,4-dinitrophenylhydrazine in 2 *N* HCl was added to 2 ml. of the supernatant thus obtained, and after standing overnight the hydrazones formed were extracted with two 4 ml. portions of chloroform-ethanol (4 : 1, v/v). The combined extracts were further extracted with 2 ml. of 10 per cent Na_2CO_3, and the carbonate solution was finally reextracted with 1 ml. of chloroform-ethanol after being acidified with 6 *N* HCl under cooling. The extract was evaporated to dryness, taken up in a small amount of ethylacetate, and subjected to an ascending paperchromatography (solvent (5): *n*-butanol-ethanol- 0.5 *N* NH_4OH, 7 : 1 : 2). The pyruvic acid DiNPH band was eluted with distilled water. An aliquot was used to determine the radioactivity in the eluate while another aliquot was subjected to colorimetry in 1 *N* NaOH at 440 mμ. The specific activity was calculated from both these values.

Determination of Pyruvate Formed—One-fourth ml. of the supernatant solution mentioned above or 2 ml. of the same for the reaction mixture without substrate was treated in a similar manner as mentioned above. The dried hydrazones obtained were dissolved in 0.2 ml. of 0.1 *M* phosphate buffer pH 7.5 and an aliquot was subjected to an ascending paper chromatography. The assay method was the same as mentioned above.

Radioactivity of Carboxyl Carbon of Pyruvate—Pyruvate-DiNPH was degraded by the method of Krebs (*4*, *6*) with a slight modification. In the main compartment of the manometric vessel was placed 1 ml. of C^{14}-pyruvate DiNPH (giving 1.203, 1.153, and 0.655×10^4 cpm for pyruvate derived from glucose-1-, -6-, and -U- C^{14}, respectively), and 2 mg. of unlabelled pyruvate DiNPH in 1.5 ml. of 0.01 *M* Na_2CO_3, in the side arm 0.36 ml. of 50 per cent H_2SO_4 and 0.75 ml. of 2 per cent $KMnO_4$, and in the center well 0.4 ml. of 20 per cent KOH. The acidic $KMnO_4$ solution was tipped in dropwise, and the reaction mixture was shaken at 37° until no more gas absorption was observed. The contents of the center well were washed out, 0.1 ml. of 10 per cent Na_2CO_3 was added, and carbon dioxide was precipitated in the form of $BaCO_3$ with 20 per cent barium acetate. The precipitate was washed twice with 15 ml. of 50 per cent ethanol, plated on a stainless steel disk, and counted with a thin window Geiger-Müller tube. Correction was made for self-absorption.

Methods for the Evaluation of Routes of Glucose Metabolism—In the application of the methods depending on the isolation and examination of pyruvate formed, theoretical values for the labelling of pyruvate can be given as illustrated in Table I, if it is assumed that pyruvate, formed via EM or ED pathway (*8*), arises equally from carbon 1–3 and

4–6, whereas that derived via HMP pathway arises exclusively from carbon 4–6. When both HMP and EM pathways are operative, the formulae in Table II, corresponding to the cases as defined below, can be constructed by reference to the distribution of carbon atoms indicated in Table I and Fig. 1, where the most commonly accepted cycle of HMP pathway is shown. That is (a) the case where carbon 2–3 do not give rise to pyruvate via HMP pathway; (b) the case where all of the carbon 2–3 do so by going through EM pathway after the first and single cycling in HMP pathway; (c) the case where the hexosemonophosphate formed via cyclic operation of HMP pathway is degraded via both HMP and EM pathway in the same proportion as the original glucose.

TABLE I

Theoretical Values for Relative Specific Activity and Distribution of C^{14} in Pyruvic Acid Formed by the EM, ED and HMP pathways of C^{14}-Glucose Metabolism

Pathway	Relative specific activity of Pyruvate to		C^{14} in pyruvate-COOH formed from	
	glucose-1-C^{14}	glucose-6-C^{14}	glucose-1-C^{14}	glucose-6-C^{14}
EM	0.5	0.5	0%	0%
ED	0.5	0.5	100%	0%
HMP	0.0	1.0	—	0%

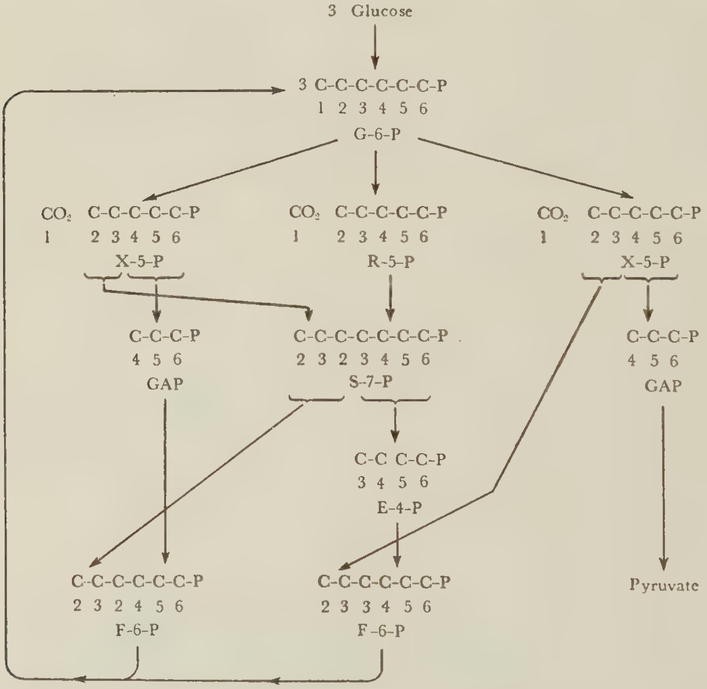

FIG. 1. The hexosemonophosphate oxidative cycle (after Wood (*3*)). Abbreviations used: G-6-P, glucose-6-phosphate; X-5-P, xylulose-5-phosphate; R-5-P, ribose-5-phosphate; S-7-P, sedoheptulose-7-phosphate; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate.

The interpretation of the data obtained from pyruvate isolated from systems metabolizing these labelled sugars is made by determining the relative specific activity of pyruvate to the substrate sugar and then ascertaining the percentage of glucose molecules degraded via HMP pathway by reference to the appropriate formula.

TABLE II

Relationship between the Relative Specific Activity of Pyruvate to Substrate Glucose and the Percentage of Glucose Molecules Oxidized via HMP Pathway When This Route and the EM Pathway alone Are Operative

Label in glucose	Percentage of glucose molecules oxidized via HMP pathway in the case of (a)	(b)	(c)
1-C^{14}	$\frac{100(1-2R)}{(1-R)}$	$\frac{300(1-2R)}{(3-R)}$	$125(1-R)-25\sqrt{25R^2-2R+1}$
6-C^{14}	$\frac{100(2R-1)}{R}$	$\frac{300(2R-1)}{R}$	$\frac{300(1-2R)}{(2-5R)}$

R: Relative specific activity of pyruvate to substrate glucose.

(a): Case where C-2 and C-3 of glucose do not yield pyruvate via HMP pathway. (b): Case where all C-2 and C-3 of glucose do yield pyruvate by going through EM pathway after the first and single cycling in HMP pathway. (c): Case where hexosemonophosphate formed via cyclic operation of HMP pathway is degraded via both HMP and EM pathway in the same proportion as the original substrate glucose.

The methods for the preparation of washed cell suspension and determination of the nitrogen content of cell materials were described in the previous paper (*1*). Glucose was determined by the colorimetric method of Park and Johnson (*7*).

RESULTS

In the absence of arsenite, pyruvate was detected only in the early stage of the oxidation of glucose by this bacterium, as described in the previous paper (*2*). In the presence of arsenite, an approximately equimolecular amount of pyruvate was produced aerobically from glucose as shown in Table III, while one more of oxygen uptake was observed for one mole of glucose consumed. (The general characteristics of arsenite inhibition will be described in our next paper).

With the arsenite-inhibited system, the specific activities of pyruvate derived from glucose-1, 6-, and U-C^{14} were determined (Table III).

A correction was made for the specific activities of the pyruvate previous to the calculation of the relative specific activities of the pyruvate to the substrate glucose and thence the participation of HMP pathway was worked out. This correction was calculated from the determined labelling present in the carboxyl group pyruvate (Table IV) on the assumption that there has been an increase in activity through fixation of metabolically produced $C^{14}O_2$.

TABLE III

Specific Radioactivity of Glucose and Pyruvic 2, 4-Dinitrophenylhydrazones Formed in the Presence of Arsenite

Label	Glucose		Pyruvate DiNPH		
	Specific activity cpm/µM	Corrected specific activity[1] cpm/µM	Formed µM	Specific activity cpm/µM	Corrected specific activity[2] cpm/µM
1-C^{14}	5.67×10^4	5.54×10^4 (±0.14[3])	4.91	2.51×10^4 (±0.06)	2.49×10^4 (±0.10)
6-C^{14}	4.66 ,,	4.57 ,, (±0.11)	4.21	2.29 ,, (±0.06)	2.33 ,, (±0.08)
U-C^{14}	3.59 ,,	2.97 ,, (±0.07)	4.92	1.46 ,, (±0.02)	1.49 ,, (±0.04)
Endogenous	—	—	0.130	—	—

Reaction mixture contained 250 µ moles of phosphate buffer, pH 7.0, 125 µ moles of $NaAsO_2$, 5 µ moles of C^{14}-glucose, 0.36 mg. N. of washed cell suspension, and water in a total volume of 2.5 ml. Shaken under air at 37° for 40–50 minutes.

1) Corrected for radiochemical purity (97.6, 98.2, and 82.8% for glucose-1, 6-, and U-C^{14}, respectively)

2) Corrected for gain in 1-C position and then for endogenous unlabelled pyruvate (see also Table IV).

3) Standard error.

TABLE IV

Determination of the Distribution of Labelling by Decarboxylation of Pyruvate Derived from Position-Labelled Glucose

Label in glucose	Pyruvate DiNPH		Total counts in $C^{14}O_2$ from carboxyl group cpm	Percentage of counts in carboxyl group %
	specific activity cpm/µM	total count cpm		
1-C^{14}	2.51×10^4 (±0.06)[1]	1.203×10^4 (±0.006)	422 472 mean 447 (±25)	3.72 (±0.24)
6-C^{14}	2.29×10^4 (±0.06)	1.153 ,, (±0.003)	161 164 mean 163 (±2)	1.41 (±0.03)
U-C^{14}	1.46 ,, (±0.02)	0.655 ,, (±0.002)	2,244 2,204 mean 2,224 (±20)	33.95 (±0.45)

Pyruvate-DiNPH was degraded by the method of Krebs (*6,4*) with a slight modification. $C^{14}O_2$ was collected in KOH and counted as $BaCO_3$. Correction for self-absorption was made.

1) Standard error.

It will be noticed that pyruvate derived from glucose-6-C^{14} contains less label in tne carboxyl group than that from glucose-1-C^{14}: this is readily explicable in terms of the decreased availability of $C^{14}O_2$ for fixation in the former case where $C^{14}O_2$ can arise only as a result of the oxidation of labelled pyruvate which has escaped the arsenite block. In fact, $C^{14}O_2$ absorbed by KOH in the center well during the course of the production of pyruvate from glucose was determined as follows; 8,400, 86, and 3,230 cpm for glucose-1-, 6-, and U-C^{14}, respectively.

Further correction is made for the production of unlabelled pyruvate from the endogenous reserves of the cell.

From the corrected specific activities of pyruvate and the substrate glucose, the percentage of glucose molecules oxidized via the HMP pathway was calculated according to the formula in Table II on the assumption that this route and/or the EM pathway were operative. The results are shown in Table V.

TABLE V

Relative Specific Activity of Pyruvate Derived from Position-Labelled Glucose and Percentage of Glucose Molecules Catabolized via HMP Pathway.

Label in glucose	Relative specific activity of pyruvate to substrate glucose[3]	Percentage of glucose molecules catabolized via HMP pathway in the case of		
		(a)	(b)	(c)
1-C^{14}	0.449 (±0.03)[2]	18.5 (10–27)[1]	12.0 (5–19)	12.2 (6–19)
6-C^{14}	0.510 (±0.03)	3.9 (0–14)	11.8 (0–45)	10.9 (0–34)
U-C^{14}	0.502 (±0.025)	—	—	—

Percentage of glucose molecules catabolized via HMP pathway was calculated by reference to the equations shown in Table II.

1) The deviation of mean values is obtained graphically from the standard error of relative specific activity.

2) Standard error.

3) Calculated from corrected specific activity shown in Table III.

DISCUSSION

The low activity in the carboxyl group of pyruvate derived from glucose-1-C^{14} might indicate a minor participation of ED system (*8*) but this is considered unlikely for the following reasons. First, the ED system is known, to date, to occur only in Gram-negative organisms (*4*, *9–13*). Secondly, the result of the degradation of pyruvate derived from glucose-6-C^{14} lends supports to the view that $C^{14}O_2$ fixation is of significance in this system. Accordingly, the operation of ED system in *Brevibacterium flavum* may be excluded. Thus, a correction for $C^{14}O_2$ fixation as well as for endogenous dilution of pyruvate was applied to the specific activities of pyruvate formed, and the corrected specific activity of pyruvate derived from glucose-U-C^{14} yielded a value for the relative specific activity, 0.502, which was in excellent agreement with the theoretical value, 0.500.

The final results quoted in Table V are obtained from the formulae in Table II(c), which assume that hexose formed by the operation of HMP cyclic pathway is degraded by both HMP and EM pathway in the same proportions as the original glucose. This seems to be a reasonable assumption and the results obtained with the two differently labelled sugars are in better agreement than if the equations in Table II(a) are used. Because of the type of the equations(c) it will be evident that glucose-1-C^{14} must give the most accurate result over the whole range of R while the use of glucose-6-C^{14} will be particularly inaccurate at the lower levels of HMP pathway participation.

We can now conclude that some 10 per cent of the substrate glucose is degraded via HMP pathway and the remainder by EM pathway in the presence of arsenite.

The above conclusion cannot be applied to the uninhibited glucose metabolism unless the use of arsenite in the experiments described is justified, since it might derange the routes of glucose metabolism occurring in the organism. Such an effect is, however, theoretically unlikely in the present instance because the inhibitor exerts its effect at a point common to all known routes of glucose metabolism. The concentration of arsenite used causes an accumulation of pyruvate but does not completely block its further metabolism, for example, the formation of α-ketoglutarate, so that, in this respect as well, the system used differs only slightly from the uninhibited one.

The evidences have been obtained for the presence of hexokinase, phosphohexose isomerase, phosphohexokinase, aldolase, triosephosphate dehydrogenase as well as glucose-6-phosphate dehydrogenase in the cell-free extracts of *Brevibacterium flavum* (*1*). This is consistent with the present isotopic data.

By using the same method, Dawes and Holms (*4*) estimated that about 30 per cent of the glucose was metabolized via HMP pathway and the remainder by EM scheme.

SUMMARY

1. Labelled pyruvic acid was produced from glucose-1-, 6-, and U-C^{14} in the presence of arsenite by washed cell suspensions of *Brevibacterium flavum* No. 2247. The relative specific activities of pyruvate to the substrate glucose were; 0.449, 0.510, and 0.502, and the percentage of counts in carboxyl group of pyruvate were; 3.72, 1.41, and 33.95 per cent, for the substrates, glucose-1-, 6-, and U-C^{14}, respectively.

2. From these results it was concluded that glucose was metabolized aerobically by both the Embden-Meyerhof and hexosemonophosphate oxidative pathway in the approximate ratio of 90 : 10.

The authors are indebted to Prof. S. Akabori of the Osaka University, Dr. T. Yamada of the University of Tokyo, and Dr. H. Oeda and Mr. N. Motozaki of our laboratory for the helpful discussion and encouragement during the course of this work.

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STUDIES ON THE PHYSIOLOGICAL CHEMISTRY OF THE SALIVARY GLANDS*

XXXVII. MOLECULAR SIZE AND SHAPE OF PAROTIN

By YOSOJI ITO, YUKIHO KUBOTA AND YASUYO SHIBUYA

(From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo)

(Received for publication, September 26, 1959)

In a previous publication (*1*), it was reported that the molecular weight of parotin was estimated to be about 800,000 from the data on the diffusion coefficient and intrinsic viscosity which were measured in a phosphate buffer of ionic strength 0.083. It is well known, however, that the diffusion coefficient gives a low value due to charge effects with protein molecules, if it is measured in the solvent of ionic strength less than 0.1. If this were the case of parotin, the value of 800,000 might be higher than that of the true molecular weight. Meanwhile, our recent experiments on the ultracentrifugal sedimentation and boundary spreading of parotin have suggested that its molecular weight may be considerably lower than that determined previously. For this reason, we have reinvestigated the molecular size and shape of parotin by the measurements of sedimentation coefficient, diffusion coefficient and intrinsic viscosity at an ionic strength of 0.2. The molecular weight of parotin found in the reinvestigation was 132,000, assuming its molecule to be a prolate ellipsoid of axial ratio of 25.7 with scarce hydration.

EXPERIMENTALS

Parotin and Solution—Parotin was purified by the fractional precipitation with ammonium sulfate from the crude preparation (the pH 5.4 precipitate), which was extracted from bovine parotid glands (*2*). Purified parotin was recrystallized four times at 12 per cent concentration of ammonium sulfate and was obtained in an electrophoretically pure form as shown in Fig. 1.

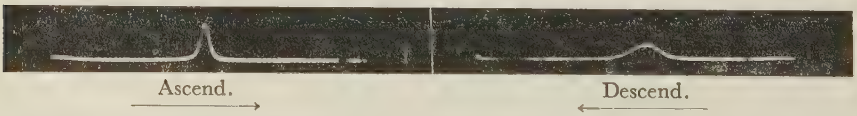

FIG. 1. Electrophoretical patterns of parotin in a citrate buffer of pH 7.6.
Protein concentration, 1 per cent; ionic strength, 0.2; voltage, 100 v.; current, 7 mA.; time, 70 minutes; temperature, 4°.

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* The title of this series "Studies on the Salivary Gland Hormones" is changed to the present one from this number.

Parotin was dissolved in a citrate buffer of ionic strength 0.2 and pH 7.6. Prior to diffusion experiments, parotin solutions were dialyzed against the same buffer for 20 hours at 0°.

Sedimentation—Sedimentation and diffusion measurements were carried out in a Spinco Model E ultracentrifuge equipped with a rotor temperature indicator and control unit (RTIC). In sedimentation experiments, the runs were made at a speed of 59,780 r.p.m. at room temperature, and the rotor temperatures were measured by RTIC. Four sedimentation experiments were carried out with protein concentrations of 0.2, 0.3, 0.6 and 1.0 g. per 100 ml. In every instance, parotin sedimented as a well defined single peak. The distance from the maximum ordinate of boundary to the reference hole was measured directly on the photographic plate with a microcomparator.

Diffusion—In diffusion experiments, the bar of the schlieren optics of the ultracentrifuge had been replaced by a phase plate. A 12 mm. synthetic boundary cell with a rubber valve was employed. The runs were carried out at a speed of 12,590 r.p.m., and the rotor temperature was maintained at 25° ± 0.1° by RTIC. At this speed, the spreading of boundary due to centrifugal force is practically negligible. Four diffusion experiments were carried out with protein concentrations of 0.3, 0.4, 0.8 and 1.4 g. per 100 ml. Diffusion coefficients were computed by the maximum ordinate-area method from the tracings which were made at fourfold linear magnification from photographs obtained with the schlieren cylindrical lens method.

Viscosity—Viscosity measurements were made with twelve protein concentrations between 0.04 and 1.0 g. per 100 ml. at 20° ± 0.02° in a capillary viscometer of Ostwald type.

RESULTS

Sedimentation Coefficient—The measured values of sedimentation coefficients were corrected to a temperature of 20° in a solvent with the viscosity and density of water by the usual procedure. The effect of hydrostatic pressure on the solvent density was not taken into account, since it may be negligible when compared with the other limitations of the accuracy. The corrected

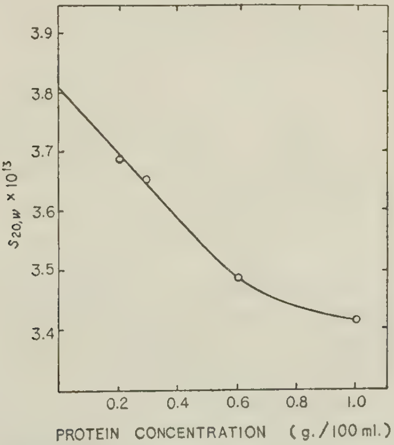

Fig. 2. Sedimentation coefficient of parotin as a function of concentration in a citrate buffer of pH 7.6.

value of the sedimentation coefficient is expressed as $s_{20,w}$. The results of sedimentation measurements are illustrated in Fig. 2, in which $s_{20,w}$ is plotted against the protein concentration. As is seen, the concentration dependence of $s_{20,w}$ of parotin is considerably strong. By extrapolating to zero concentration, the value of $s_{20,w}$ at infinite dilution is found to be 3.81 S. At concentration below 0.2 g. per 100 ml., the reciprocal of $s_{20,w}$ changes linearly in relation to protein concentration, and the identical value of $s_{20,w}$ at infinite dilution is obtained by extrapolating the $1/s_{20,w}$ *versus* concentration line to zero concentration. This may indicate that the interaction among molecules decreases at low concentrations. At concentrations of less than 0.1 g. per 100 ml., however, the boundaries were not sufficiently sharp to obtain accurate values of $s_{20,w}$, which resulted in the deviation of the plots of $1/s_{20,w}$ *versus* concentration from the straight line drawn by the method of least squares. The partial specific volume, V, used for the calculation of $s_{20,w}$ was measured at 20°±0.02° in a pycnometer (2 ml. in volume) of Ostwald type, and was found to be 0.753 ml. per g. This value is in good agreement with that determined previously (*1*).

Diffusion Coefficient—The observed values of diffusion coefficients were corrected to 20° in a solvent with the viscosity of water by the usual procedure. The corrected value is expressed as $D_{20,w}$. The results of diffusion measurements are shown in Fig. 3. Extrapolating the curve to zero concentration, the value of $D_{20,w}$ at infinite dilution is found to be 2.84×10^{-7} cm.^{2} per second. The concentration dependence of $s_{20,w}$ and $D_{20,w}$ may be ascribed to the interaction among parotin molecules in solutions.

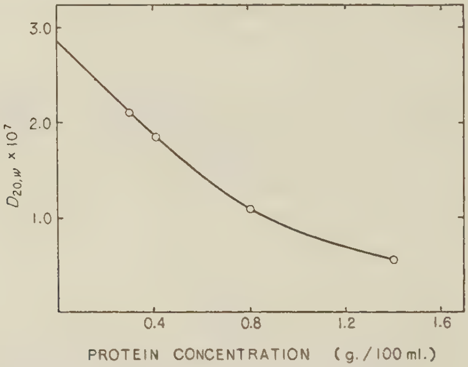

FIG. 3. Diffusion coefficient of parotin as a function of concentration in a citrate buffer of pH 7.6.

Intrinsic Viscosity—The reduced viscosity calculated from the observed relative viscosity was plotted against the protein concentration, and a straight line was obtained as shown in Fig. 4. By extrapolating to zero concentration, the value of the intrinsic viscosity, $[\eta]$, is found to be 0.428. The volume fraction intrinsic viscosity, ν, is calculated to be 56.84 by the following formula: $\nu=100\,[\eta]/V$.

Frictional Ratio and Axial Ratio—The frictional ratio of the molecule calculated from the values of $s_{20,w}$ and $D_{20,w}$ was 2.21. The axial ratio was computed from the frictional ratio according to the procedures of Oncley (*3*) and Perrin (*4*), assuming the molecule to be an ellipsoid and the amount of hydration to be 0 or 0.3 g. per gram protein. The results are summarized in Table I. In the last column of the table, the molecular dimensions are calculated from the values of the axial ratio and molecular weight.

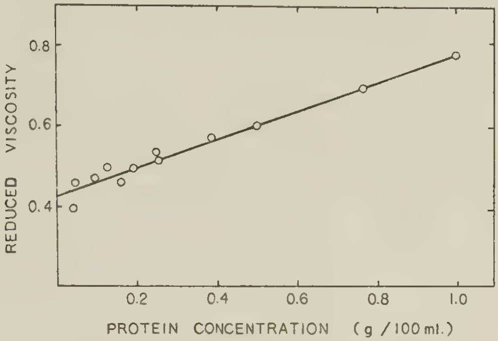

FIG. 4. Reduced viscosity *versus* concentration curve for parotin in a citrate buffer of pH 7.6.

TABLE I

Axial Ratio and Molecular Dimensions of Parotin Calculated from $s_{20,w}$ and $D_{20,w}$

Molecular shape	Hydration (g. water per g. protein)	Axial ratio	Molecular Dimensions (Å)
Prolate	0	25.7	592×23
ellipsoid	0.3	19.4	492×25
Oblate	0	39.7	6×232
ellipsoid	0.3	27.9	7×206

On the other hand, the axial ratio of the molecule of parotin was also calculated from the volume fraction intrinsic viscosity by Simha's formula (*5*). The corresponding frictional ratio was computed from the value of the axial ratio by Perrin's formula (*4*). In the case where the hydration of the molecule was taken into account, the calculation of the axial ratio and frictional ratio was made according to the procedure of Oncley (*3*). In this calculation, we assumed that the amount of hydration water of the molecule was 0 or 0.3 g. per gram protein. The results are listed in Table II.

Molecular Weight—The molecular weight of parotin was calculated by combination of sedimentation, diffusion and viscosity data following the usual treatments. The molecular weight, M_{sD}, calculated from $s_{20,w}$ and $D_{20,w}$ was 132,000. The molecular weight, $M_{s\nu}$, was calculated from $s_{20,w}$ and ν, and the molecular weight, $M_{D\nu}$, from $D_{20,w}$ and ν, assuming the molecular shape and the amount of hydration water. The results are listed in Table II.

TABLE II

Axial Ratio, Frictional Ratio and Molecular Weight of Parotin Calculated from Intrinsic Viscosity

Molecular shape	Hydration (g. water per g. protein)	Axial ratio	Frictional ratio	$M_{s\nu}$ 1)	$M_{D\nu}$ 2)
Prolate	0	25.4	2.20	132,000	132,000
ellipsoid	0.3	20.6	2.26	137,000	122,000
Oblate	0	82.6	2.79	188,000	65,000
ellipsoid	0.3	58.4	2.79	188,000	65,000

1) The molecular weight calculated from the sedimentation coefficient and intrinsic viscosity.

2) The molecular weight calculated from the diffusion coefficient and intrinsic viscosity.

Amount of Hydration Water—It may be seen from these results that nearly the same values for the molecular size are obtained by three different methods, if we assume the unhydrated molecule of a prolate ellipsoid. It is generally

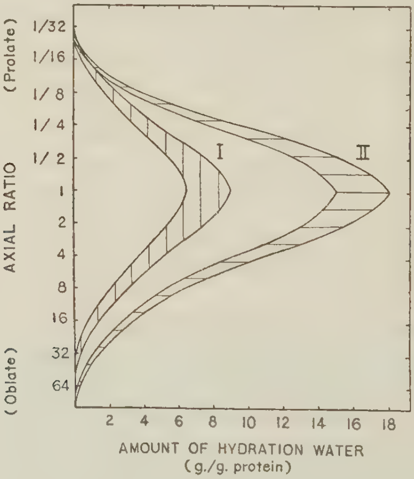

FIG. 5. Combination of amount of hydration water and corresponding axial ratios of parotin calculated from $s_{20,w}$, $D_{20,w}$ and ν assuming experimental errors. I represents area calculated from $s_{20,w}$ and $D_{20,w}$ assuming errors of ±5 per cent, and II from ν assuming error of ±10 per cent.

accepted, however, that a gram of protein contains average 0.3 g. of hydration water in aqueous solution, and therefore the nonhydration of the molecule of parotin may be somewhat incomprehensible. For this reason, we have computed the minimum and maximum amounts of hydration water of the molecule of parotin according to the procedure of Oncley (*3*), assuming the experimental errors of $s_{20,w}$ and $D_{20,w}$ to be ±5 per cent and that of ν to be ±10 per cent. The results are shown in Fig. 5. The dotted area on which two curves cross represents the possible combination of the amount of hydration water and the corresponding axial ratios of the molecule of parotin. It may be inferred from this computation that the amount of hydration water is 0–1.0 g. per gram protein if we take into account the experimental errors, and that the molecule of parotin is a prolate ellipsoid rather than an oblate ellipsoid or sphere.

DISCUSSION

The value of 132,000 for M_{sD} is in good agreement with those for $M_{s\nu}$ and $M_{D\nu}$, if it is assumed that the molecule of parotin is a prolate ellipsoid without hydration. In other cases, $M_{s\nu}$ and $M_{D\nu}$ deviate considerably from M_{sD} as shown in Table II. Since M_{sD} is calculated directly from the sedimentation and diffusion coefficients without any assumption pertaining to the molecular shape and the amount of hydration, it would be the most reliable value for the molecular weight compared with $M_{s\nu}$ and $M_{D\nu}$. Hence, we would adopt the value of 132,000 for the molecular weight of parotin. The frictional ratio of 2.21 determined from $s_{20,w}$ and $D_{20,w}$ agrees well with that evaluated from ν on the same assumption as above. In that case, the corresponding axial ratio may be calculated to be 25.7. From these data, the length of the major axis and that of the minor axis of the molecule are calculated to be 592 Å and 23 Å, respectively.

The amount of hydration of the molecule of parotin is estimated to be 0–1.0 g. per gram protein, if we assume that the experimental errors of $s_{20,w}$ and $D_{20,w}$ are ±5 per cent and that of ν is ±10 per cent. The values of the experimental errors, however, may be far smaller than those assumed, and, in fact, the amount of hydration would be so scarce that its effect on the molecular size may be negligible.

The molecular weight of parotin determined previously by diffusion and viscosity measurements was 800,000, which is about six times higher than is reported in this paper. The previous value (2.21×10^{-7}) of the diffusion coefficient used for the calculation of the molecular weight is somewhat lower than was determined in this investigation, and that may be one of the reasons why an unusually high molecular weight was obtained previously. As is well known, the diffusion coefficient gives a low value, if it is measured in a solvent of low ionic strength (less than 0.1). This phenomenon may be caused by the mutual interaction among protein molecules due to electrical charges. Since the previous diffusion measurements were carried out in a phosphate buffer of ionic strength 0.083, the low value for the diffusion coefficient may have been obtained on account of this charge effect. It remains uncertain, however, whether this low value is due to the charge effect only, or is due also to changes of the molecular size such as the association of parotin molecules. Although we have not observed the association of parotin molecules in the citrate and phosphate buffers over a wide range of protein concentrations, it may occur under suitable conditions of pH, ionic strength, etc. In order to clarify these problems, investigations are now being carried out concerning the molecular kinetics of parotin under various conditions.

SUMMARY

The molecular size and shape of parotin were investigated by sedimentation, diffusion and viscosity measurements, and the following results were obtained.

1. The molecular weight of parotin was determined to be 132,000 from the sedimentation coefficient (3.81×10^{-13}), diffusion coefficient (2.84×10^{-7}) and intrinsic viscosity (0.428), assuming the molecule to be a prolate ellipsoid with scarce hydration.

2. The frictional ratio and the corresponding axial ratio of the molecule were calculated to be 2.21 and 25.7, respectively. From these data, the molecular dimensions were evaluated to be 592 Å × 23 Å.

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STUDIES ON THE DISTRIBUTION AND PHOSPHATE-TURNOVER OF THE ACID-SOLUBLE PHOSPHORUS COMPOUNDS IN VARIOUS NORMAL AND NEOPLASTIC TISSUES OF RATS

II. COMPARISON OF THE CHROMATOGRAMS OBTAINED WITH VARIOUS TISSUES INCLUDING TUMORS

By SHIGEO HORIE

(*From the Department of Biochemistry, Faculty of Medicine, University of Tokyo, Tokyo*)

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In spite of the enormous efforts made by many investigators for finding out a metabolism specific for neoplastic tissues, sufficient knowledge was not obtained in past decades of years (*1*). Although this problem is very complicated and difficult to study, it must be investigated more extensively because of its fundamental importance for human life and its biological significance.

As described previously (*2*), the author and Terada worked out a modified semi-micro gradient elution chromatography suitable for analysing a small amount of specimen. So, as a kind of screening for cancer research, a comparative study of the distribution and phosphate-turnover of the acid-soluble phosphorus compounds was intended with the use of the modified method. As a whole, no definite qualitative difference between normal and neoplastic tissues could be found. However, besides the known fact, that is, the relatively active uptake of inorganic phosphate by tumor tissue, some additional findings could be obtained. In addition to these, various tissue-specific distribution- and turnover-patterns of the acid-soluble phosphorus compounds in normal tissues were observed.

The results of these analytical experiments will be reported in the present paper.

EXPERIMENTALS

The preparation of tissue extracts and the semi-micro column chromatography with the use of Dowex 1 formate were described in the previous paper (*2*). Normal tissues

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abbreviations;

AMP, ADP, ATP: adenosine mono-, di, and triphosphate
GMP, GDP, GTP: guanosine mono-, di-, and triphosphate
CMP, CDP, CTP: cytidine mono-, di-, and triphosphate
UMP, UDP, UTP: uridinc mono-, di-, and triphosphate
DPN: diphosphopyridine nucleotide

were obtained from the rats of Wistar-King A strain as described previously. For tumor bearing animals, usual albino rats were purchased commercially because the strain rats were relatively resistant to the implantation of tumors. The tumors used are AH 7974 and AH 130 strains of ascites hepatoma supplied from the Sasaki Research Institute*. These tumors had been induced from liver tissues by feeding azo dyes, and transplanted from rats to rats. The AH 130 strain grows more rapidly and is more sensitive to X-ray irradiation than the AH 7974 strain. In this work, the ascites tumor cells were injected subcutaneously into the back of the animals**. The tumor cells continued to grow there and formed solid types of tumors. The excised tumor tissues were usually colorless. These tumors were analysed 8 to 14 days after the transplantation. When any sign of local necrosis was found, the tumor was omitted from experimental materials.

Injection of P^{32} inorganic phosphate and other treatments of the animals were described in the previous paper (*2*).

RESULTS

The Distribution of the Ultraviolet Absorbing Materials in the Acid-Soluble Extracts from Various Tissue—Analyses of the U. V. absorbing materials in the acid-soluble extracts from various tissues were carried out with the modified semi-micro gradient elution chromatography. In these analyses, the P^{32} injection was not carried out. The intensity of the U. V. absorption of each peak was calculated on the chromatogram. Although there was no verification to conclude that each of these peaks was composed from a single nucleotide, it was considered possible to regard the absorption value of a peak as that of a nucleotide and to compare various tissues as far as each nucleotide contributed to the major part of the absorption of each peak. The results of the analyses are summarized in Table I. The total absorption values $\sum E_{260}$ of acid-soluble nucleotides per g. of wet tissue in liver, muscle and heart ranged from 13 to 17, and the values in the tissues other than these three ranged from 6 to 9. Per cent of the absorption of adenosine phosphates in total nucleotides was 40 to 60 in the majority of tissue, but relatively high percentage of 78.5 was observed in muscle. The ratio GTP+UTP/ATP was in the range from 0.1 to 0.3 in the majority of tissues, but very low ratio of 0.02 in muscle and relatively high ratio of 0.45 in thymus were observed. Tumors are known to contain relatively low concentration of DPN. In this study, the liver cell carcinomas also showed low DPN values compared with liver tissue notwithstanding the fact that the former had the fairly resembled pattern of nucleotide distribution to the latter. The values of DPN in the liver cell carcinomas were also relatively low compared with other tissues except for testicles.

The Distribution and Phosphate-Turnover of the Acid-Soluble Phosphorus Compounds—As already shown (*3*), when P^{32} is injected into an animal, the radioactivity distributes at different rates into various tissues of the animal as the time passes. For example, the distribution into liver tissue is relatively fast and the distribution into bone tissue is slow. In addition to this, the incorporation of P^{32} into various phosphorus compounds in a given tissue occurs at different rates. For example, the incorporation into inorganic phosphate or ATP is fast and the incorporation into DPN or AMP is slow. So, for comparing strictly the phosphate-turnover of various phosphorus compounds in various tissues and for understanding of tissue-specific patterns of phosphate metabolism, numbers of analyses must be run along the specific activity curve of total phosphorus with each tissue. This requires enormous number of experiments and is practically very difficult to be carried out. Thus, in this report, it was decided to compare various tissues at an adequately fixed time after the intraperitoneal injection of P^{32}. If this fixed time after the injection is too short, very poor incorporation will result with some tissues and the turnover pattern of various compounds can not be obtained. On the contrary, if the fixed time after the injection is too long, a tendency of equal distribution of P^{32} into various phosphorus compounds

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* Kanda, Chiyoda-ku, Tokyo.

** Kindly assisted by Dr. Shigeyoshi Masuyama of the Department of Radiology, Faculty of Medicine, University of Tokyo.

TABLE I

The Distribution of Acid-soluble Nucleotides in Various Tissues of Rats

Tissue	Total Nucleotides $\sum E_{260}$/g. wet tissue	per cent Adenosine Phosphates	Ratio GTP+UTP /ATP	DPN $\sum E_{260}$/g. wet tissue
Liver 1	15.58	43.9	0.192	1.046
Liver 2	17.09	39.6	0.150	0.852
Liver 3	14.81	47.4	0.188	1.228
Muscle	15.49	78.5	0.021	0.390
Heart	13.58	62.6	0.100	1.054
Brain	7.61	49.9	0.305	0.452
Kidney	7.52	41.7	0.193	0.794
Lung	8.69	52.2	0.302	0.387
Spleen	7.17	68.2	0.189	0.355
Thymus	9.17	52.5	0.450	0.516
Testicle	7.74	68.0	0.160	0.199
Whole Fetus	3.61	33.2	0.268	0.462
Fetal Liver	7.50	64.0	0.197	0.784
Liver Cell Carcinoma (AH7974)	6.50	38.0	0.253	0.172
Liver Cell Carcinoma (AH7974)	5.75	57.6	0.133	0.176
Liver Cell Carcinoma (AH 130)	8.77	40.8	0.326	0.254

$\sum E_{260}$: sum of the absorption of each tube at 260 mμ.

will result in some tissues such as liver. With reference to the results appeared in literature and those obtained from the authors' preliminary experiments, it was decided to analyse mainly one hour after the intraperitoneal injection of 60 μc of P^{32}. Fig. 1 to Fig. 15 illustrate the results of the analyses of perchloric acid extracts from various tissues.

Fig. 1 and 2 are the chromatograms obtained with liver. Fig. 1 shows the result of analysis carried out one hour after the intraperitoneal injection of P^{32}. Fig. 2 shows that carried out 15 minutes after the injection of P^{32}. Brumm, Potter, and Siekevitz (*4*) already reported the chromatogram showing 15 minutes after the injection of P^{32}. All these three show essentially the same distribution pattern of the radioactivity. U. V. Absorption peak 1 to 19 agreed mostly with those described previously (*4*, *2*). Radioactivity peak A in Fig. 1, the Dowex 1 formate unadsorbable fraction, was found to be composed from phosphoryl choline and a phosphorus compound containing amino acid. Peak C was composed mainly from glucose-6-phosphate, and peak D from L-α-glycerophosphate (*2*).

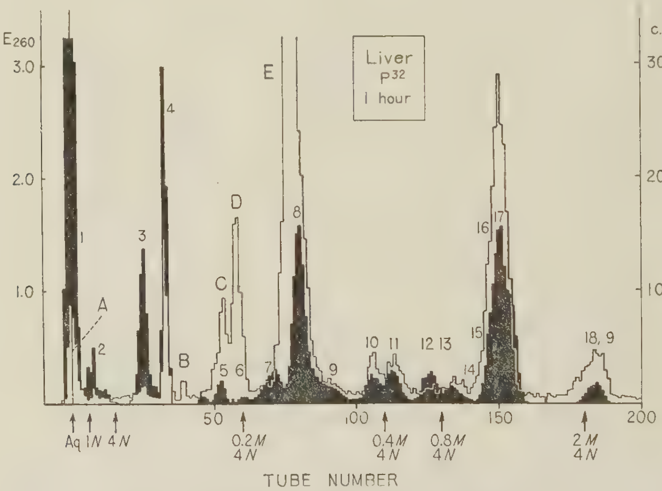

FIG. 1. Chromatography of the acid-soluble extract from rat liver.

rat: 120 g., male, liver tissue analysed: 2.7 g. One hour after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

black peaks: U. V. absorption, colorless peaks: radioactivity 3: DPN, 4: AMP, 5: GMP, 6: CDP, IMP, 7: UMP, 8: ADP, 10: UDPAG, 11: UDPG, 16, 17: UDP, ATP, 18, 19: GTP, UTP.

A: unadsorbable radioactivity, C: G6P, D: L-α-glycero-P, E: inorganic phosphate.

Fig. 3 illustrates a chromatogram obtained from muscle. Presence of a high radioactive peak B, and also of peak C, D and F were the characteristic feature of this chromatogram. The positions of peaks B, C and D corresponded to those of peaks B, C and D in liver, respectively. But, whether these peaks were composed from the identical substances with the cases in

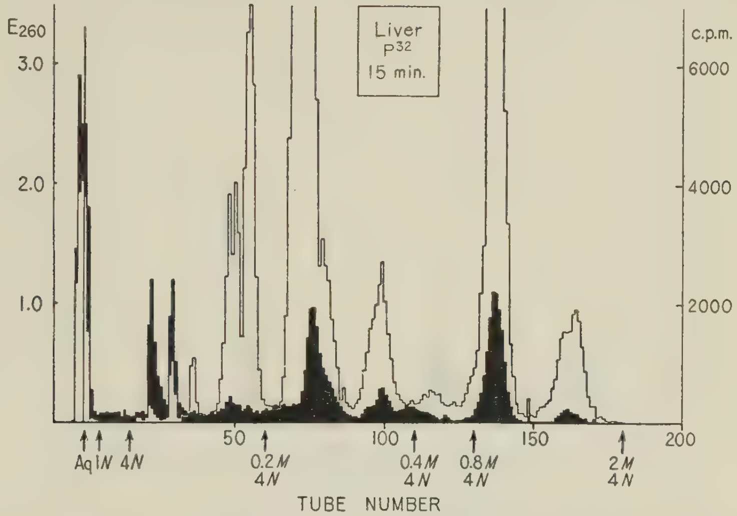

FIG. 2. Chromatography of the acid-soluble extract from rat liver.
rat: 90 g., male, liver tissue analysed: 2.25 g. 15 minutes after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.
black peaks: U. V. absorption, colorless peaks: radioactivity.

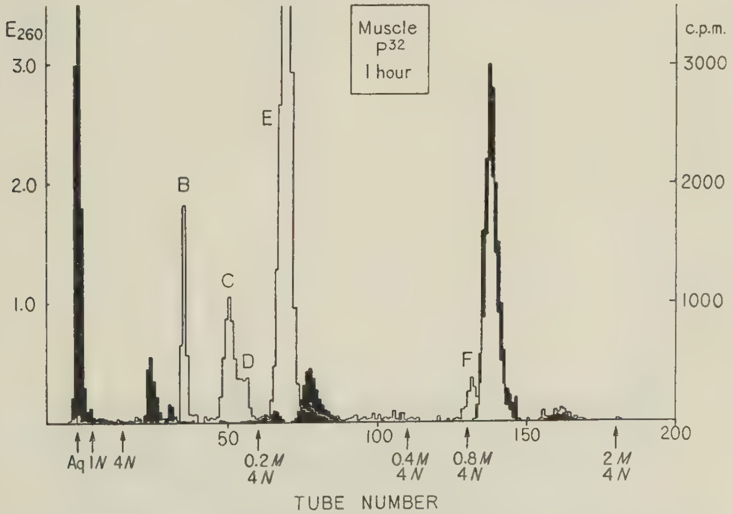

FIG. 3. Chromatography of the acid-soluble extract from rat muscle.
rat: 100 g., male, muscle tissue analysed: 1.75 g. 1 hour after the intraperitoneal injection of P^{32} inorganic phosphate.
black peaks: U. V. absorption, colorless peaks: radioactivity.
For individual peaks, see text.

liver or not, was not studied precisely. Peak F appeared just in front of ATP and had no U. V. absorption. It was supposed that this might be

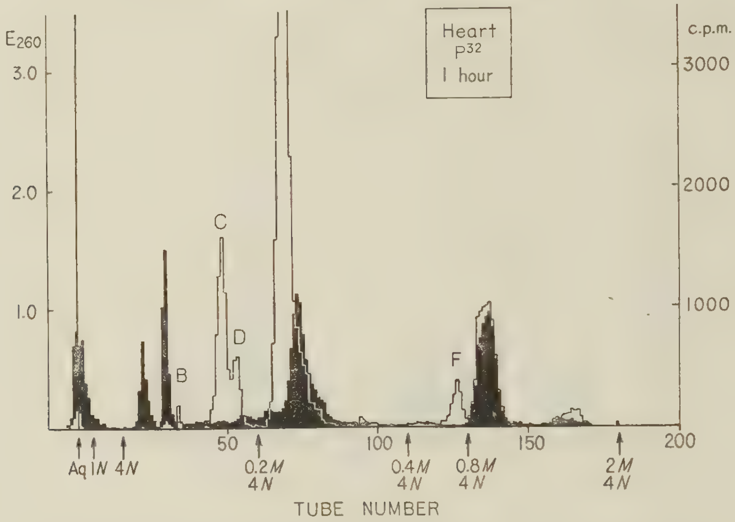

Fig. 4. Chromatography of the acid-soluble extract from rat heart.

rats: 120 g. and 160 g., males, heart tissue analysed: 1.4 g. 1 hour after the intraperitoneal injection of P^{32} inorganic phosphate.

black peaks: U. V. absorption, colorless peaks: radioactivity.

For individual peaks, see text.

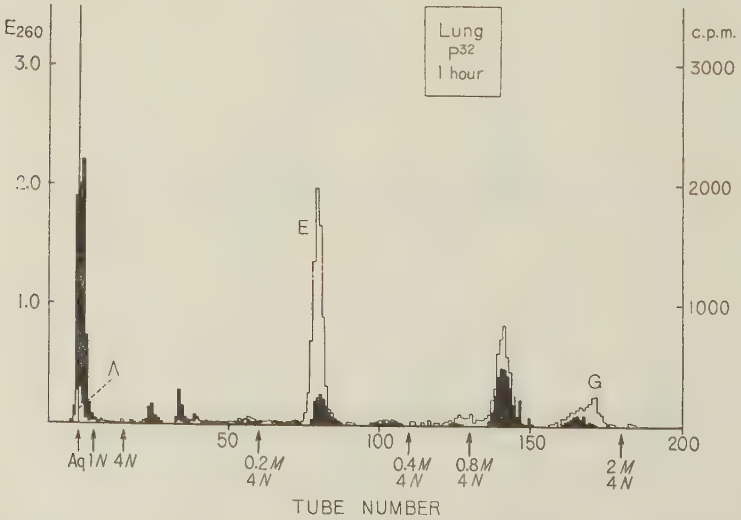

Fig. 5. Chromatography of the acid-soluble extract from rat lung.

rat: 120 g., female, lung tissue analysed: 1.05 g. 1 hour after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

colorless peaks: radioactivity, black peaks: U. V. absorption.

For individual peaks, see text.

hexose diphosphate, but the further analysis was not carried out.

Fig. 4 illustrates a chromatogram obtained from heart. The distribution of the radioactivity of P^{32} showed a considerable resemblance to that of muscle. But, differing from that of muscle, peak B was low.

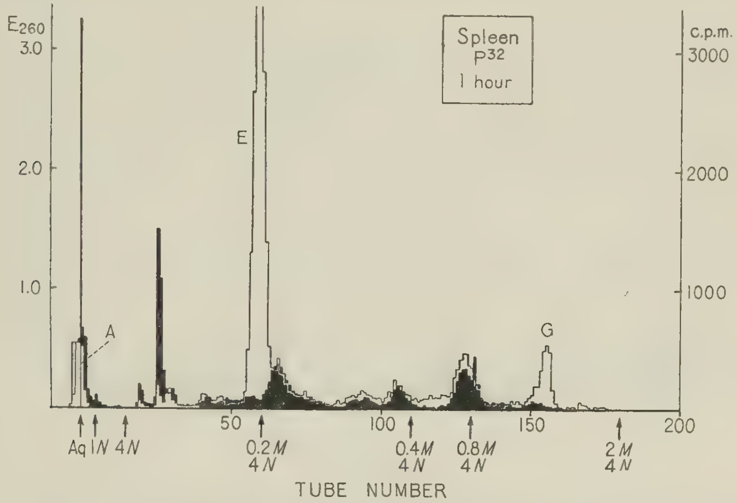

FIG. 6. Chromatography of the acid-soluble extract from rat spleen.
rats: 180 g. and 240 g., males, spleen tissue analysed: 1.5 g. 1 hour after the intraperitoneal injection of 60 μc each of P^{32} inorganic phosphate.
black peaks: U. V. absorption, colorless peaks: radioactivity.
For individual peaks, see text.

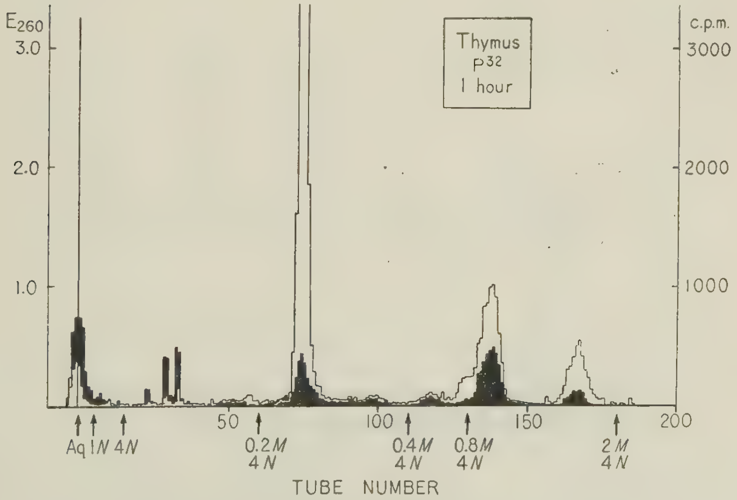

FIG. 7. Chromatography of the acid-soluble extract from rat thymus.
1 g. of thymus tissue was obtained from 4 male rats weighed 80 g. one hour after the intraperitoneal injection of 30 μc of P^{32} inorganic phosphate into each rat.

Fig. 5 shows a chromatogram from lung, and Fig. 6 from spleen. These two showed relatively poor incorporation of P^{32} into organic phosphate except for ATP, peak A, and peak G. Peak G was supposed to be 2,3-diphosphoglyceric acid, but strict identification was not carried out. Peak A in Fig.

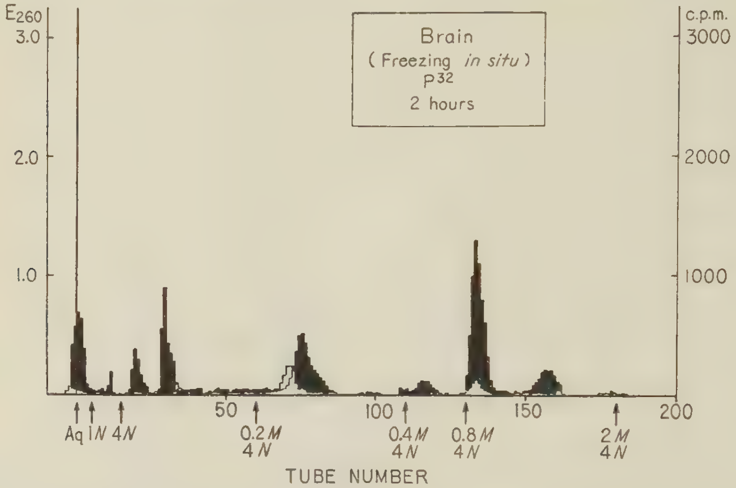

FIG. 8. Chromatography of the acid-soluble extract from rat brain.
rats: 70 g. and 80 g., females, brain tissue analysed: 2 g. Brains were obtained by freezing *in situ* 2 hours after the intraperitoneal injection of 30 μc of P^{32} inorganic phosphate.

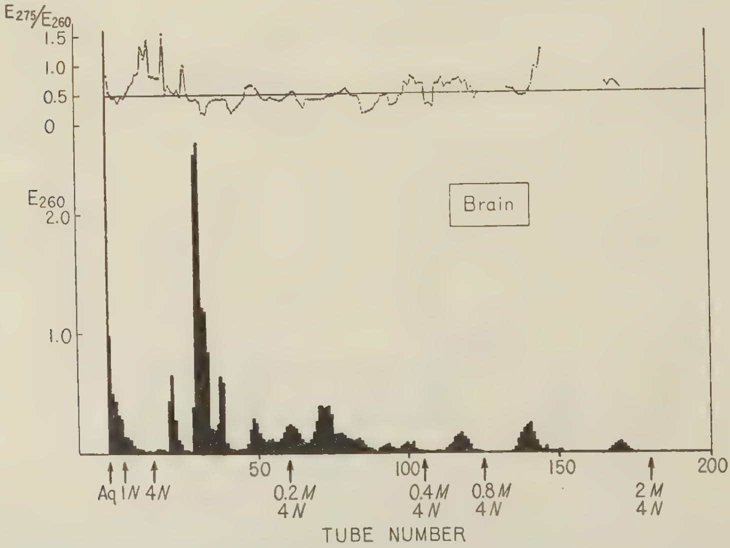

FIG. 9. Chromatography of the acid-soluble extract from rat brain.
3 g. of the brain tissue was obtained from two male rats weighed 180 g.

6 was proved to be composed from the same substances as in the case of liver.

Fig. 7 shows a chromatogram obtained with thymus. High incorporation of P^{32} into nucleotide triphosphate, especially into GTP and UTP in relative to ATP, was the characteristic feature of this tissue. Radioactivity of peak A was also high.

Fig. 8 and 9 are the chromatograms obtained with brain. In Fig. 8, the rats were freezed *in situ* with liquid nitrogen two hours after the injection of P^{32}. Only very low incorporation into the brain was observed. Fig. 9 illustrates the chromatogram of the brain which was obtained after usual decapitation followed by the freezing of the tissue in acetone-dry ice. No P^{32} injection was carried out in this case. As shown in this figure, extensive decomposition of nucleoside triphosphate occurred when freezing of the whole animal with liquid nitrogen was omitted. So far as tested, brain and kidneys were the tissues which showed this kind of rapid decomposition of triphosphates.

Fig. 10 illustrates the results obtained with testicles. This chromatogram shows the poor distribution of P^{32} and low incorporation of P^{32} into organic phosphates.

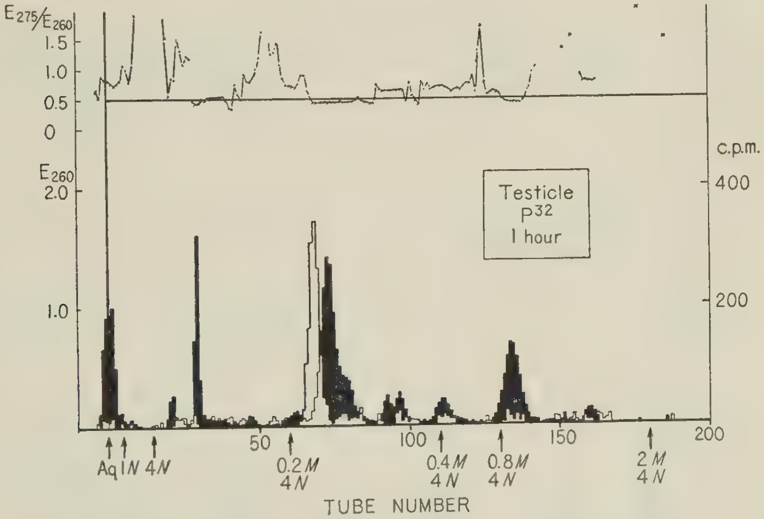

Fig. 10. Chromatography of the acid-soluble extract from rat testicles. rat: 200 g., male, testicle tissue analysed: 2.5 g. 1 hour after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

In Fig. 11, a case of analysis of fetal liver is shown. The distribution of P^{32} into this tissue was relatively low, but the incorporation into organic phosphates was remarkable compared with the total distribution of P^{32}. In this chromatogram the incorporation pattern of P^{32} shows essentially the same characteristics as that of adult liver.

Fig. 12 represents a chromatogram obtained with a solid type of ascites hepatoma (AH 130) one hour after the intraperitoneal injection of P^{32}. The

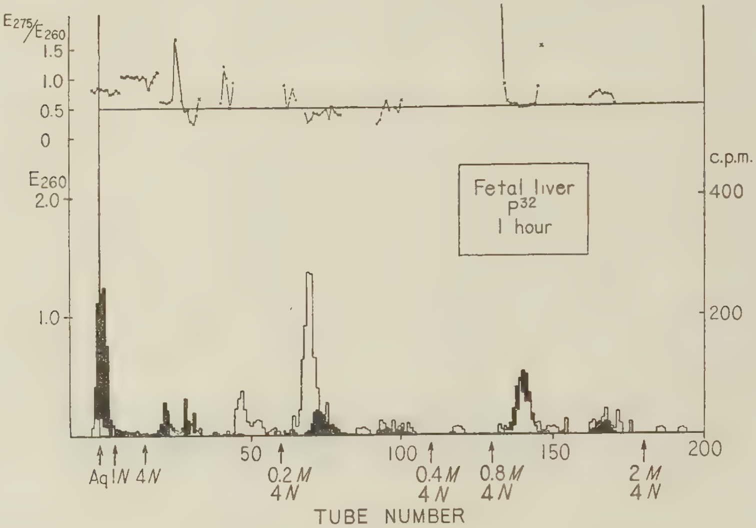

FIG. 11. Chromatography of the acid-soluble extract from liver of rat fetus.

0.95 g. of fetal liver tissue was obtained from 7 fetuses (littermate) 1 hour after the injection of P^{32} inorganic phosphate into the peritoneal cavity of the mother.

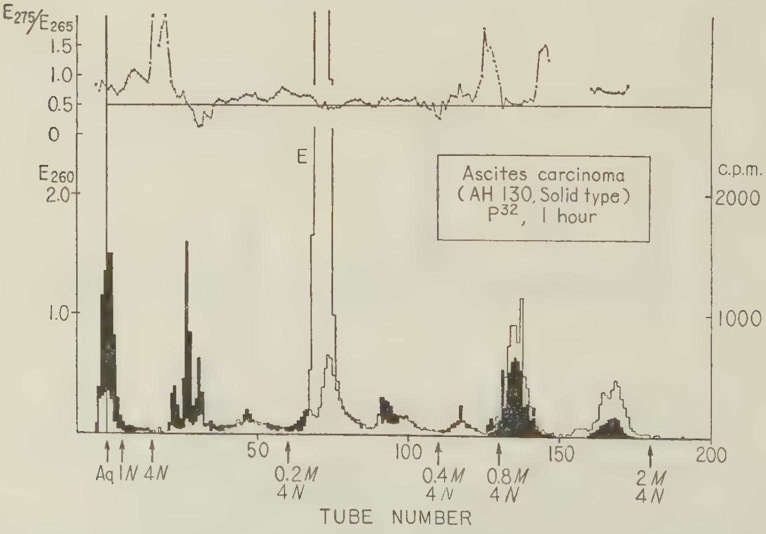

FIG. 12. Chromatography of the acid-soluble extract from the solid type of ascites carcinoma (AH 130).

rat: 200 g., male, carcinoma tissue analysed: 2.8 g. 1 hour after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

distribution pattern of U. V. absorbing materials in the tumor showed considerable resemblance to that of liver. But the distribution pattern of the radioactivity showed remarkable difference. In the tumor tissue, relatively

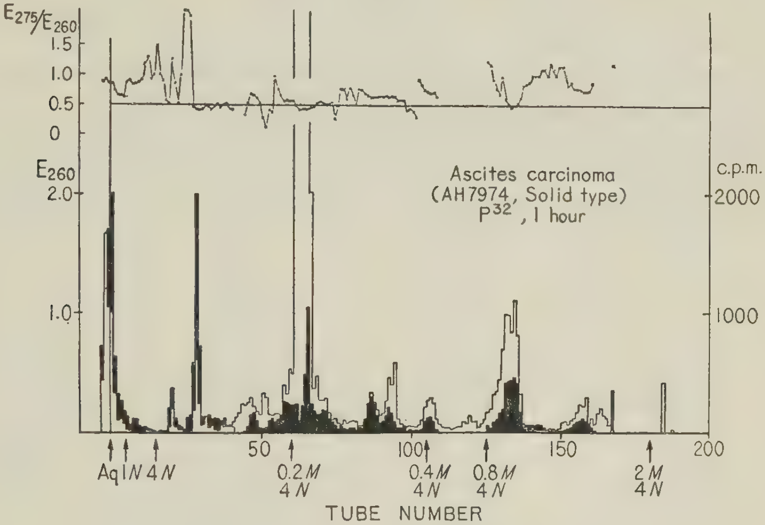

FIG. 13. Chromatography of the acid-soluble extract from the solid type of ascites carcinoma (AH 7974).

rat: 80 g., male, carcinoma tissue analysed: 2.8 g. 1 hour after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

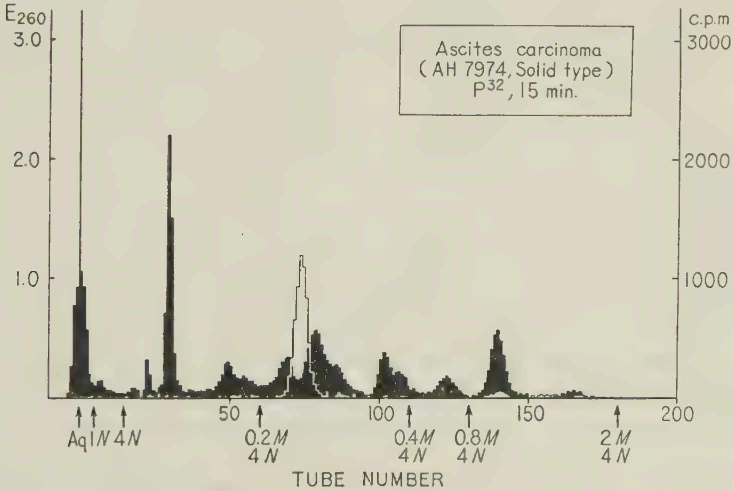

FIG. 14. Chromatography of the acid-soluble extract from the solid type of ascites carcinoma (AH 7974).

rat: 200 g., male, carcinoma tissue analysed: 2.8 g. 15 minutes after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

low incorporation into organic phosphate as a whole was observed in spite of the high radioactivity of the inorganic phosphate peak. Only peak A and nucleotide triphosphates showed appreciable radioactivity. Peak C and D were very low, or in some cases, absent in the tumor. In these respects, the distribution pattern of the radioactivity showed resemblance to those of lung, spleen or thymus.

Fig. 13 shows a chromatogram obtained with AH 7974. Almost the same tendency as in the case of AH 130 was observed.

Fig. 14 shows pattern of AH 7974 tumor 15 minutes after the injection, and Fig. 15 shows that 3 hours after. At 15 minutes, only inorganic phosphate was radioactive. After 3 hours, a tendency of equal distribution of P^{32} into nucleotides was observed.

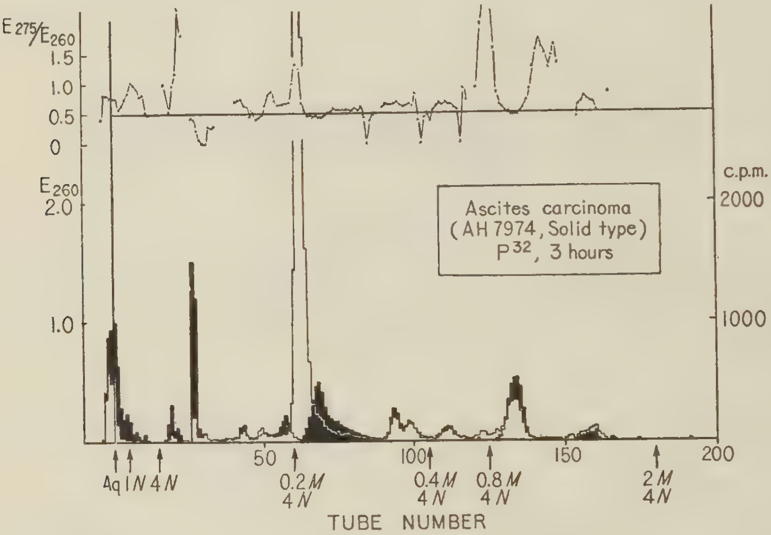

FIG. 15. Chromatography of the acid-soluble extract from the solid type of ascites carcinoma (AH 7974).

rat: 120 g., male, carcinoma tissue analysed: 2.6 g. 3 hours after the intraperitoneal injection of 60 μc of P^{32} inorganic phosphate.

As already known, tumor tissues show the high uptake of the injected radioactive P^{32}. By the above experiments, a large part of this P^{32} was shown to present as inorganic phosphate.

Table II summarizes these results. Per cent of radioactivity of inorganic phosphate in the total acid-soluble radioactivity ranged 35 to 55 in various normal tissues but ranged over 70 in tumor tissues. Unfortunately, the absolute amount of acid-soluble radioactivity in various tissues could not be compared directly in Table II, because the body weight of the animals used and the total weight of the tumor mass varied considerably.

This extraordinary high incorporation of P^{32} inorganic phosphate in tumor tissue was suppressed remarkably by local X-ray irradiation. The suppression reached maximum about 3 hours after the irradiation, and then gradually disappeared (*5*).

TABLE II

The Radioactivity of the Inorganic Phosphate in Various Tissues of Rats One Hour after the Intraperitoneal Injection of P^{32}

Tissue	Radioactivity of Inorganic Phosphate ($\sum$cpm./g. wet tissue)	Radioactivity of Total Acid-soluble Phosphates ($\sum$cpm./g. wet tissue)	per cent Radioactivity of Inorganic Phosphate in the Total Acide-soluble Phosphates
Liver	13,744	35,773	38.5
Muscle	12,420	26,526	46.8
Heart	19,120	34,601	55.4
Lung	6,896	15,972	43.1
Spleen	11,092	23,129	48.0
Thymus	18,020	37,382	48.2
Testicle	561	989	56.8
Fetal Liver	1,156	3,228	35.9
AH 130	17,979	24,942	72.0
AH 7974	36,257	47,721	76.0

$\sum$cpm.: sum of the radioactivity of each tube.

DISCUSSION

The Distribution of the U. V. Absorbing Materials—It must be mentioned that the values of $\sum E_{260}$/g. calculated for each tissue showed considerable agreement and dispersed in a relatively narrow range. It seems to be also worth to mention that liver, muscle and heart contained almost twice as much amounts of nucleotides as the other tissues. As the values of $\sum E_{260}$ calculated for various tissues performing respective functions showed relatively little variety, the total amount may be presumed to be under the control of some more fundamental cellular mechanism. But, at present, there is no experimental basis for discussing this mechanism.

On the other hand, in Table I, it was also shown how much the content of the individual nucleotides actually vary from tissue. Under the settled experimental conditions, these distribution of the nucleotides in a given kind of tissue from different rats showed almost the same pattern except for small variations. Thus it is apparent that the distribution pattern of nucleotides is maintained steadily by some biological controlling mechanisms in respective tissue cells.

As to the tumor tissues, they had clearly different pattern compared with liver tissue from which they were induced. The difference was the decreased amount of acid-soluble U. V. absorbing metarials per g. of wet tissue, especially of oxidized DPN. However, this must be evaluated in comparison with other normal tissues. The patterns of the tumors are supposed to be different from those of liver mainly because of their dedifferentiation, and also of their transformation into reproductive tissue. So, it is dangerous to regard all the differences as the signs related directly to malignancy. This kind of mistake can be avoided by comparing carefully with many tissues including normal reproductive tissues. The low content of oxidized DPN in tumor tissues has been discussed by many, but, from the reason mentioned above, further careful examinations are necessary. The content of oxidized DPN in these tumors ranged in the lower limit of those in normal tissues, but not so much lower than normal testicle.

The Distribution of P^{32} Radioactivity—From the reason discussed above, the comparison between liver and liver carcinoma should be made here again with reference to other tissues. The distribution pattern of injected P^{32} in the carcinoma tissue showed some distinct differences from that in liver tissue. One of these is that the radioactivity of glucose-6-phosphate and of α-glycerophosphate in the tumor tissue was far less than that in liver tissue. Unfortunately, there is no direct experimental basis for discussing the meanings of this difference. The tumors might have some alterations in the steady flow of carbohydrate metabolism. This high radioactivity peaks of glucose-6-phosphate and α-glycerophosphate were observed only in liver, fetal liver, muscle, and heart, and not observed in spleen, lung, thymus, *etc.* Consequently, it is reasonable to regard the absence of these peaks in the tumor tissue not as a direct expression of the malignancy but as a result of dedifferentiation of tumor cells.

By the way, it was unexpected to find out the very high radioactivity of α-glycerophosphate in liver, muscle and heart. In liver, this may have some connection with lipid metabolism but further investigation is necessary for clarifying the actual meanings. Liver, muscle and heart are known to contain L-α-glycerophosphate dehydrogenase. However, it is not yet clear that the radioactive α-glycerophosphate was formed whether by the action of the dehydrogenase or by the action of glycerokinase.

Next, the attention should be given to the fact that the considerable incorporation of P^{32} into Fraction A was always observed in liver, spleen, thymus, tumor *etc.* but very few in muscle and heart. This fraction was presumed to have some relation to the reproductive ability, and is under further investigation.

Among the results reported in this paper, those which could be assumed to have some possibility of being specific character of tumor was the high percentage of the radioactivity of inorganic phosphate in the total acid-soluble radioactivity. In this case, the radioactive inorganic phosphate was not resulted from ATP through the action of ATPase, because the specific activity of inorganic phosphate was higher than that of ATP and also, even in the very early stage after the injection of P^{32}, inorganic phosphate showed higher radioactivity than that of ATP. It seems to be natural to postulate that the tumor consumed inorganic phosphate very rapidly for some reason. What is the reason can not be answered here. The reason might be either the requirement of inorganic phosphate by actively glycolysing tumor cells or the active and arbitrary consumption by the altered cell membrane. As the tumor tissues were almost colorless, they could not contain large amounts of highly radioactive circulating blood. There is an another possibility, that is, the extracellular fluid in the tumor tissue might contain large amouts of radioactive inorganic phosphate. But this last possibility seems to have not so much reliability.

Metabolic Studies with Column Chromatography in Combination with Tracer Techniques—Concerning the metabolism in living organisms fairly concrete knowledges have been accumulated in literature. A complicated and large but static map of the chemical reactions including vast fields of metabolism are now established. Many of these reaction systems are known to do occur generally in various organisms. However, the relative rate of metabolism of each reaction may vary more or less from tissue to tissue, and relatively little is known about these differences. Physiological and pathological differences between normal and malignant tissues, young and old tissues, or stimulated and degenerated tissues, might be understood at least in part as the changes in the relative rate of the metabolism or, in other words, as the shift of the steady flow of metabolisms.

For studying these dynamic patterns of metabolism in intact tissues, various methods can be available. As one of the approaches, extensive column chromatography of metabolites in combination with the use of labelled compounds will give a powerful clue. But, of course, this type of analysis has some limitations.

The extensive performance of the chromatography is very troublesome and tedious. But, if automatic equipments for analysis and recording are properly used, this fault will be overcome. After the intermediary metabolites are identified on the chromatogram, the experiments can be carried out routinely under suitable schedule.

This type of analysis can properly applied only when the cell population is not so complicated. This is also one of the limitations. When needed, the method of synchronous division of cells must be applied.

The problem of permeability of cell membrane is also difficult to solve. The metabolites which arise in the cell are not always permeable to the cell wall, so the use of tracers will be limited. This difficulty will be partly reduced by using the derivatives which can easily penetrate into the cell and be restored to the original metabolite within the cell.

The decomposition of the metabolites or coenzymes may occur during the course of the analysis, so this possibility must also be kept in mind.

The most serious limitation of this type of analysis is the fact that this gives no knowledge concerning the intracellular localization of the metabolism. Although the same types of analysis can be performed with isolated cellular particles, the results obtained by such experiments have no verification that they are physiological and have no direct relation to the actual intracellular metabolic rate. So far as intact cells are dealt with, the intracellular topography of the metabolic rates must be studied with the use of micro-spectrophotometry or so (*6*).

At any rate, this types of analysis will bring much yield if they are applied in combination with other suitable methods.

Next, one more point must be discussed here. The intraperitoneal injection of P^{32}, adopted in this report, may cause the staining of intraperitoneal organs to be analysed. Rinsing of the organs before the homogenization may reduce the contamination. But the rinsing was not applied because it wastes much time and bring about an extensive degradation of nucleotides.

SUMMARY

1. Using a modified semi-micro gradient elution chromatography, the distribution of the acid-soluble nucleotides in various normal and neoplastic tissues of rats was compared and the variations of the distribution were described.

2. The distribution and phosphate-turnover of the acid-soluble phosphorus compounds were also studied by intraperitoneal injection of P^{32} followed by the chromatographic analysis. The distribution patterns of nucleotides and radioactivity in liver, muscle, heart, lung, thymus, spleen, testicles, brain, fetal liver and experimental hepatomas were illustrated and the differences between these tissues were pointed out.

3. The characteristics of the experimental hepatoma tissue as compared with the normal liver tissue were as follows: a) The concentration of oxidized DPN was low. b) The incorporation of P^{32} inorganic phosphate into glucose-6-phosphate and L-α-glycerophosphate was absent or, if any, very low. c) Per cent of radioactivity of inorganic phosphate in the total acid-soluble radioactivity was extraordinary high as compared with other tissues as well as the liver tissue.

The author is deeply indebted to Prof. N. Shimazono for his kind advice and encouragement.

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PHOSPHORUS METABOLISM OF FUNGAL CELLS

I. OXIDATIVE PHOSPHORYLATION BY MITOCHONDRIA FROM ASPERGILLUS ORYZAE

By KOZO IWASA

(From the Department of Biology, Faculty of Science, University of Osaka, Osaka)

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In the previous papers, it was reported that mitochondrial particles isolated from *Aspergillus oryzae* have magnesium activated ATP*-ase activity and this is increased by thermal ageing and by preincubation with magnesium and calcium (*1*). They can also oxidize all intermediates of the tricarboxylic acid cycle except pyruvate and can oxidize reduced DPN, to oxygen through cytochrome c (*2*).

Recently, Utter, Keech and Nossal (*3*) succeeded in obtaining an active preparation of mitochondria from yeast which was capable of oxidative phosphorylation.

In this communication, mitochondrial particles catalyzing phosphorylation coupled with oxidation of succinate are described. Their activity under various conditions and with inhibitors was investigated.

MATERIALS AND METHODS

Culture of Aspergillus oryzae:—Dried conidiospores were inoculated on to the surface (*4*) of a medium containing 15 g. sucrose, 5 g. ammonium nitrate, 0.5 g. primary potassium phosphate, 1 g. magnesium sulfate, a trace of ferric chloride and 1 liter of tap water in flat aluminium vessels. After incubation at 30° for 40 hours, the mycelial mats were floated in water, blotted and weighed.

Preparation of Mitochondria:—Thirty-five g. of the mycelial mats at 0° and 170 ml. of a medium containing 0.5 *M* sucrose, and 0.01 *M* EDTA with or without 0.006 *M* potassium citrate was homogenized in a homogenizer (Nihon Seiki Kogyo Co. Japan) for 15~25 sec. (full speed) at 0~3°. The homogenate was filtered through gauze and centrifuged at low speed ($900 \times g$) for 5 minutes to remove debris. Then the supernatant was recentrifuged at high speed ($17,500 \times g$) for 5 minutes. The precipitate was suspended in incubation medium before use. The amount of suspension used for one test contained mitochondria equivalent to 2~4 g. of wet mycelial mats.

Nucleotide Complex from Asp. oryzae:—150 g. (wet weight) of the mycelial mats were put into 150 ml. of *N* perchloric acid and pressed to obtain an acid soluble extract. Extraction with 0.5 N perchloric acid was repeated twice more. In the combined extract of ca. 500 ml., free nucleotides were adsorbed on charcoal (Norit Extra treated with octanol) (*5*), separated from the acid, washed with water, and eluted by 50 per cent ethanol adjusted with ammonia to pH 9.5. The eluate, containing free nucleotide but not sugar phosphate ester or *i*P was dried *in vacuo* over H_2SO_4. It was dissolved in water to 10 ml. before use. (0.1 ml. of the nucleotide solution was equivalent to 1.5 g. of mat).

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* The following abbreviations are used in this paper: ATP, adenosine triphosphate: EDTA, ethylenediaminetetraacetate; DPN, diphosphopyridine nucleotide; DNP, dinitrophenol; *i*P, inorganic phosphate.

Assay Methods:—Oxygen consumption was measured by the Warburg technique using trimethylpentane as the manometer fluid. Inorganic phosphate was determined according to the method of Fiske and Subbarow (*6*). Inorganic P^{32} incorporated into organic compounds was assayed according to the method of Nielsen and Lehninger (*7*) with slight modifications. Radioactivity was measured in a Geiger-Müller tube with a mica end-window.

RESULTS

Activity of Mitochondria Isolated by Different Media—Mitochondria prepared by use of a medium containing 0.5 M sucrose, and 0.01 M EDTA (preparation medium A) possess lower oxidative phosphorylation activity than when 0.006 M potassium citrate (preparation medium B) was added to the medium (Table I). Moreover on ageing in suspension at 5° for 2 hours, mitochondria

TABLE I

Oxidative Phosphorylation and Preparation Media

Preparation medium	Substrate added	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
A	None	0.03	0.00	—
A	Succinate 20 μmoles	1.00	1.66	0.60
B	None	0.41	0.34	—
B	Succinate 20 μmoles	1.38	1.54	0.90
After 2 hours at 5°				
A	None	0.05	0.18	—
A	Succinate 20 μmoles	0.09	1.38	0.06
B	None	0.22	0.30	—
B	Succinate 20 μmoles	0.88	1.32	0.67

Preparation medium A; 0.5 M sucrose, 0.01 M EDTA, pH 7.2 with KOH. Preparation medium B; 0.5 M sucrose, 0.01 M EDTA, and 0.006 M K-citrate, pH 7.2 with KOH. Reaction system in the Warburg vessel was as follows: Main chamber; 10~15 μmoles *i*P (radioactivity of iP^{32} is 5,000~10,000 c.p.m. per μmole), 5 μmoles $MgCl_2$. 2 μmoles ATP, 10 μmoles KF, 20 μmoles glucose, hexokinase (able to react with 2~5 μmoles of ATP for 10 minutes under these condition), DPN, 0.5 mg. per vessel, and water to 0.8 ml. Side arm; 0.2 ml. of mitochondrial suspension (equivalent to 2~4 g. of fresh mycelia). Centre well; 0.1 ml. of 40% KOH. Temperature, 25°. Reaction period, 30 minutes.

were damaged faster in medium A than B. When mitochondrial pellets were first precipitated at high speed and then resuspended and reprecipitated, their activity was decreased.

Effect of Inorganic Phosphate Concentration—The mitochondrial preparation contained very little *i*P in the free state (less than 2 μg. per test reaction). As shown in Fig. 1, the oxidative and phosphorylative activity varied with the concentration of *i*P, *i.e.* both increased proportionally to a maximum at 10 μmoles *i*P per ml. though the P/O ratio was scarcely altered.

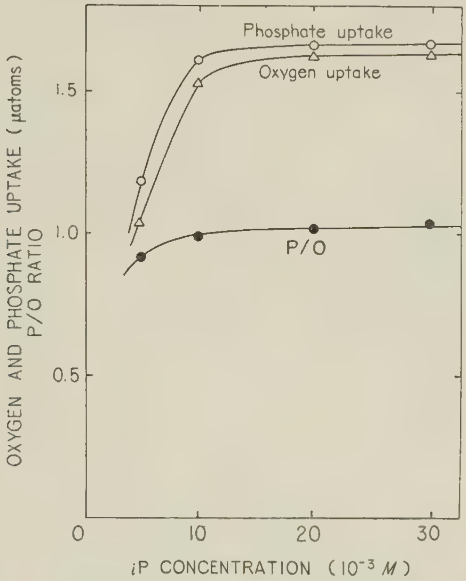

FIG. 1. Effect of *i*P concentration on the oxidative phosphorylation.

Preparation medium, B. 40 μmoles of succinate were added as oxidative substrate. Other experimental conditions were as same as Table I.

Effect of Hexokinase—In yeast mitochondria, the fact that oxidative phosphorylation could not be detected without addition of hexokinase, may be due to the phosphatases activity of the preparation (*3*). In fungal mitochondria, it was found that phosphorylative activity was increased with the hexokinase concentration. The oxidative activity was slightly increased, and consequently the P/O ratio only increased gradually (Fig. 2).

Using succinate as substrate the P/O ratio without added hexokinase was about 0.7, whereas with sufficient hexokinase it about 1.4. Therefore phosphatases such as ATP-ase (*1*) and glucose-6-phosphatase seem to be present in low concentration in mitochondrial preparations of *Aspergillus oryzae*.

Effect of pH—According to Utter *et al.* (*3*) the optimum pH for the P/O ratio of yeast mitochondrial preparations is about 6.6. Our fungal mitochondrial preparations has a relatively wide pH range between 6.0 and 7.2 for phosphorylation. The optimal pH of the reaction medium, however, for oxidation and phosphorylation was about pH 6.6 and that for the P/O ratio was about pH 7.0, as shown in Fig. 3.

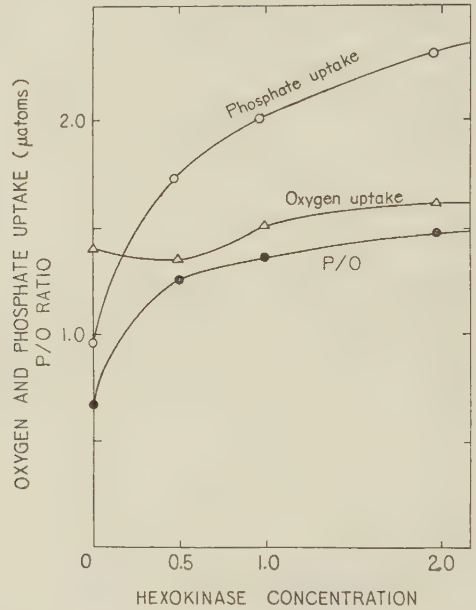

Fig. 2. Effect of hexokinase concentration on oxidative phosphorylation.

Preparation medium; B. 40 μmoles of succinate were added as substrate. 0.1 ml. of hexokinase solution, contains ca. 100 μg. of the enzyme protein reacting with 5 μmoles of ATP for 10 minutes at 30° was added. Other experimental conditions were as in Table I.

Recently Slater *et al.* (*8*) have found in animal mitochondria the interesting fact that the pH optimum curve for oxidative phosphorylation has three peaks at pH 6.3, 7.4, and 8.5. These correspond to the phosphorylation steps of the electron carriers: that is the oxidase, cytochrome b and DPNH oxidase respectively. With fungal mitochondria, when the pH curve plotted against the P/O ratio with succinate as substrate, there are two peaks at pH 6.3 and 7.0. This is similar to Slater's observation.

Effects of Nucleotide Complex, DPN and Cytochrome c on Oxidative Phosphorylation—In animal mitochondria, it has been found that oxidative phosphorylation activity is lost by removal of nucleotides such as ATP and coenzyme A (*9*). The activity of aged mitochondria could be restored by addition of DPN (*10*). Oxidative phosphorylation in animal mitochondria is known not to require the addition of cytochrome c, unlike that of higher plants (*11*). It was demonstrated that in *Asp. oryzae* mitochondria phosphorylation coupled with oxidation of succinate was increased by added nucleotide complex and DPN as shown in Table II. These additions hardly increased oxidation. The effect of nucleotide complex on the P/O ratio was proportional to its concentration.

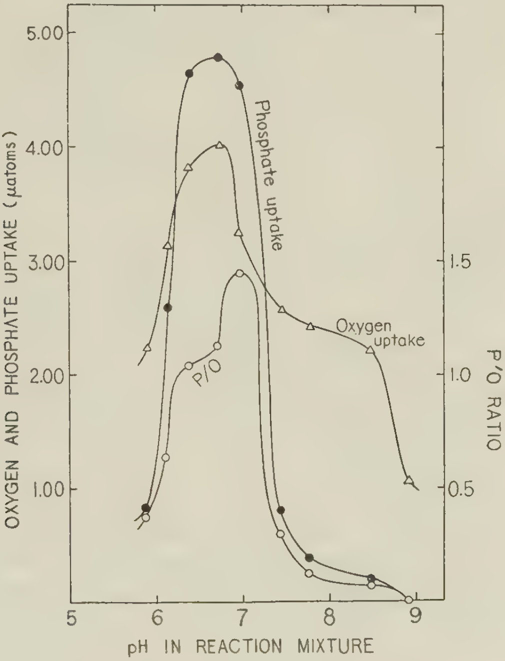

Fig. 3. Effect of pH on oxidative phosphorylation. The reactions were started by addition of the mitochondrial suspension to the reaction mixture adjusted to the required pH with *N* KOH.

Since, as described above (Table I), the mitochondria from *Asp. oryzae* have labile phosphorylative activity, the effect was probably due either to prevention for ageing or to a restoration of lost activity, or both. This will be discussed in the following report.

Oxidation of succinate was increased by added cytochrome c but phosphorylation was not altered, and consequently the P/O ratio was lowered.

Inhibition by Antimycin A—It has been well known that antimycin A inhibits electron transport by blocking cytochrome b. Thus oxidative phosphorylation is stopped.

Phosphorylative activity itself is probably not inhibited by antimycin A because the ATP-*i*P exchange reaction which was presumably a part of the oxidative phosphorylation reaction is not inhibited by antimycin A (*12*). Furthermore, it has been found that the oxidation pathway in mitochondria not involving phosphorylation is not sensitive to this reagent. As shown in the Table III, the P/O ratio of our preparation was only slightly decreased by addition of antimycin A, but oxidation and phosphorylation were strongly inhibited.

TABLE II

Effect of Nucleotide complex on Oxidative Phosphorylation

Substrate added	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
None	0.31	0.63	—
Succinate	1.62	1.60	1.01
,, +cytochrome c	1.54	1.94	0.80
,, +DPN	1.89	1.50	1.58
,, +nucleotide comlex	2.22	1.67	1.62
,, +nucleotide complex+cytochrome c	2.14	2.06	1.04
None	0.98	0.48	—
Nucleotide complex, 0.1 ml.	1.00	0.72	1.39
Succinate	2.77	2.04	1.36
,, +nucleotide complex, (0.05 ml.)	3.27	2.21	1.48
,, +nucleotide complex (0.1 ml.)	3.49	2.34	1.49
,, +nucleotide complex (0.2 ml.)	3.82	2.30	1.66

Succinate; 40 μmoles. Cytochrome c; 0.01 μmoles. DPN; 0.5 mg. Preparation medium; B. Other experimental conditions as in Table I.

TABLE III

Effect of Antimycin A on Oxidative Phosphorylation

Succinate added (40 μmoles)	Antimycin A added (μg.)	Phosphate uptake (μmoles)	Oxygen consumption (μatoms)	P/O ratio
—	—	0.22	0.21	(1.05)
+	—	1.12	1.10	1.02
+	0.25	0.28	0.27	1.04
+	0.5	0.18	0.20	0.90
+	1.0	0.12	0.14	0.86
+cytochrome c	1.0	0.16	0.27	0.59

Preparation medium; B. Other experimental condition as in Table I.

Oxygen consumption was slightly increased by addition of cytochrome c. From these results, it is concluded that the decrease of phophorylation is caused by inhibition of electron transport by antimycin A, and that the oxidative pathway of succinate which is sensitive to reagent is only a minor pathway.

Inhibition of DNP—2,4-Dinitrophenol, which is known to inhibit phosphorylation and promote oxidation, inhibited phosphorylation in mitochondria of *Asp. oryzae* (Fig. 4).

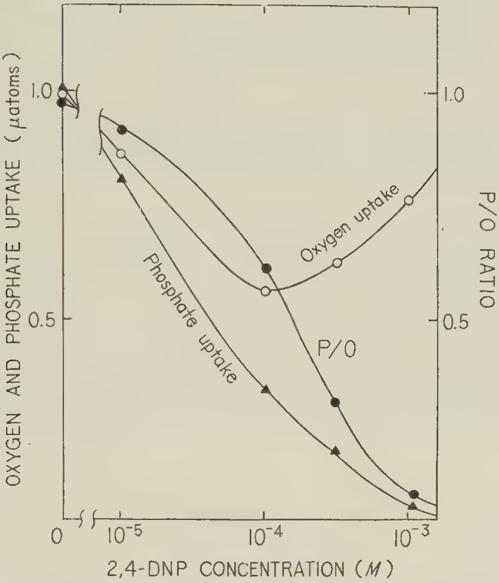

FIG. 4. Inhibition of oxidative phosphorylation by 2,4-DNP. For details see text.

Up to 10^{-4} *M*, inhibition of oxidation and phosphorylation was proportional to the concentration of reagent. The P/O ratio decreased gradually with the concentration of reagent due to decreased phosphorylative activity. At a concentration of more than 10^{-4} *M*, DNP obviously acted as an uncoupler.

DISCUSSION

From microscopic observations the mitochondria of *Aspergillus oryzae* are abundant in young cells of aerated mycelia. If the nature of the mitochondria is affected by changes in the environment of the cells, their nature must be different from those of yeast mitochondria. In support of this, a P/O ratio of as high as 1.0 to 1.6 was observed with phosphorylation coupled with the oxidation of succinate, while with yeast mitochondria it was generally between 0.6 and 0.9 (*3*). Moreover, the mitochondrial preparation contains scarcely any *i*P or phosphatases, or oxidative enzymes which are insensitive to antimycin A. From the results, it seems probably that a preparation isolated by a medium containing potassium-citrate has very fresh, intact mitochondria.

Phosphorylative activity is promoted by addition of a nucleotide complex obtained from the mats of *Asp. oryzae*. Moreover, phosphorylative activity of the mitochondria is lost faster when the preparation medium contains no EDTA, probably because of the calcium in homogenate as known in animal mitochondria (*13*). From these results it seems that on ageing the mitochondrial preparation easily liberated some components which were essential for phosphorylative activity. Some of these components may have been nucleotides. The pH optimum curve of the P/O ratio is similar to that of animal mitochondria.

The fundamental function and mechanism of the fungal mitochondria in oxidative phosphorylation seems to be similar to that of animal, higher plant and yeast mitochondria. However this stability with regard to phosphorylation is apparently less.

An uncoupling effect of 2,4-DNP on oxidative phosphorylation was seen clearly at the somewhat high concentration (2×10^{-3} M of DNP). At a lower concentration, inhibition of phosphorylation was in parallel with that of oxidation, but the P/O ratio was only slightly lowered. Antimycin A inhibited phosphorylation and respiration and lowered the P/O ratio slightly. It may be that the phosphorylation mechanism of fungal mitochondria consists of reaction steps which are more closely linked together than those of animal mitochondria.

SUMMARY

1. Mitochondria prepared from aerated mycelia of *Aspergillus oryzae* in 0.5 M sucrose containing 0.01 M EDTA catalyzed the phosphorylation coupled with oxidation of succinate.

2. The phosphorylative activity was stabilized by addition of K-citrate.

3. High concentration of *i*P and hexokinase in the reaction mixture increased the phosphorylative activity. Addition of *i*P increased oxidation but hexokinase did not.

4. The oxidative phosphorylation had two pH optima at pH 6.3 and 7.0.

5. Addition of a nucleotide complex prepared from the organism and of DPN stimulated oxidative phosphorylation (as judged by the P/O ratio).

6. The inhibitory effect of antimycin A and 2,4-DNP was studied. Antimycin A-insensitive oxidation of this preparation was very small and the phosphorylation mechanism, is tightly coupled with oxidation.

The author wishes to express his thanks to Prof. K. Okunuki for his valuable guidance. Thanks are also due to his colleagues Mr. T. Higashiyama of the same laboratory, and Dr. F. Imamoto of the laboratory of pharmacology, faculty of medicine, Osaka University, for their help and discussion.

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ON THE PREPARATION AND PROPERTIES OF 2,4,6-TRINITROPHENYL-AMINO ACIDS AND -PEPTIDES*

By TSUNEO OKUYAMA and KAZUO SATAKE

(From the Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Tokyo)

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The FDNB method of Sanger (*1*) has been used extensively during the past decade; not only for the N-terminal analysis of peptides and proteins (*2*, *3*), but also for an assay or identification of amino-acids (*2*, *3*, *4*), -alcohols (*2*, *3*), -sugars (*5*) and -lipids (*6*), for a method of molecular weight determination (*7*) and for a partial labeling of protein in connection with the biological essentiality of different groups (*8*, *9*). For the latter special circumstances, however, other nitrophenyl reagents, such as the water soluble DNBS (*9*, *10*, *11*) or the slower reacting 2,4,5-trinitrotoluene (*12*), seem to possess more advantages.

In course of our studies on partial labeling reactions of protein, TNBS was found to be an interesting TNP reagent (*13*). The sulfonic acid, quite different from FDNB (*1*, *2*, *3*) or ClTNB (*13*), reacted with primary amino group but neither with water, phenolic hydroxy- nor imidazole imino-groups. The selective TNP-lation of amino group occurred with a moderate speed in water solution at pH 8 and at room temperature, thus under far milder conditions than those of the DNP-lation with DNBS (*9*, *10*, *11*). Since the first paper on TNP-amino acid by Hirayama (*14*), there have been only isolated reports on the TNP-lation with ClTNB (*13*, *14*, *15*, *16*) but nothing on the TNP-lation with TNBS nor on the properties of TNP-amino acids. So in this papar, we wish to report the TNP-lation of amino acids and peptides with the both reagents, ClTNB and TNBS, and some properties of the resulting TNP derivatives, which seem to be necessary for the further studies on the TNP-method in protein chemistry.

EXPERIMENTALS

Materials—ClTNB was prepared from picric acid by the treatment with phosphorous pentachloride (*17*) or with tosyl chloride (*18*). m. p. 83°.

TNBS was obtained as monohydrate by the treatment of ClTNB with sodium bisulfite, followed by the recrystallization from *N* hydrochloric acid (*19*). m.p. 180° (dehydrated at 100°).

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* The following abbreviations are used: DNP, 2,4-dinitrophenyl; ClDNB, 1-chlor-2,4-dinitrobenzene; FDNB, 1-fluor-2,4-dinitrobenzene; DNBS, 2,4-dinitrobenzene 1-sulfonic acid; TNP, 2,4,6-trinitrophenyl; ClTNB, 1-chlor-2,4,6-trinitrobenzene; TNBS, 2,4,6-trinitrobenzene 1-sulfonic acid. Abbreviation for amino acids or the residues, are those suggested by Brand and Edsall (*Ann. Rev. Biochem.*, **16**, 224 (1947)).

The following compounds were used for TNP-lation: Gly, L-Ala, L-Val, L-Leu, L-Ileu, L-Ser, L-Thr, L-Asp, L-Glu, L-Met, L-Cys, L-Lys, L-Arg, L-His, L-Phe, L-Tyr, L-Try and L-Pro from Ajinomoto Co. Ltd.; L-cysteic acid prepared by the performic acid-oxidation of L-Cys; Gly-Gly, Gly-DL-Val, Gly-DL-Ser, L-Leu-L-Tyr, DL-Ala-DL-AspNH$_2$, L-Pro-Gly and Gly-Gly-DL-Ala from California Corporation for Biochemical Research; oxidized beef insulin A and B prepared by the method of Sanger (*20*).

Analytical Methods—Elementary analyses were kindly done by Analytical Center, Pharmaceutical Institute, University of Tokyo.

Ultraviolet absorption spectra were measured in 1.00 cm. silica cuvettes by the use of Hitachi Spectrophotometer, Type DU-2.

Infrared absorption spectra of TNP-amino acids dispersed in potassium bromide disk, were recorded with Koken Infra-Red Spectrophotometer, Type D-101.

Paper chromatographic analysis of TNP-amino acids was done on Toyo Filter Paper No. 51 by ascending method; using *n*-butanol saturated with 5 per cent ammonia as the first solvent and 1.5 *M* sodium phosphate (pH 5.4) as the second. As the solvent for ether-insoluble TNP-amino acids, a mixture of *n*-butanol, acetic acid and water (4 : 1 : 2 *v/v*) was also used. The intense yellow color of TNP-amino acids (more than 1 μg.) allowed them to be located easily on paper chromatograms and to be identified from their *Rf* values. For the quantitative analysis, each spot was eluted from the paper with a definite volume of aqueous solution of 4 per cent sodium bicarbonate, separately and estimated at their light absorption maximum (350 mμ.), spectrophotometrically. As the standard, the molar extinction coefficient of the corresponding authentic TNP-amino acid was used. The procedures were just similar to those of DNP-amino acid, which have been discussed in the reviews by Ohno (*2*) and by Fraenkel-Conrat (*3*).

Free guanidino-, imidazole imino- and phenolic hydroxy-groups unreacted with TNP reagents, were assayed spectrophotometrically, using Sakaguchi-(*21*), Pauli's diazo-(*22*) and Millon-(*23*) reactions, respectively. The substitution of these groups with DNP (also with TNP) group caused no coloration with Sakaguchi-(*24*), Pauli-(*13*, *25*) and Millon-(*26*) reactions, respectively.

RESULTS

I. TNP-lation with ClTNB—As shown in the following example, the TNP group of ClTNB was introduced into the amino group of amino acids or lower peptides and partially into water molecule, in the 66 per cent alcoholic solution at pH 5 to 8 and at room temperature; thus under the conditions which had been reported to be optimum for the DNP-lation with FDNB (*1*, *2*, *3*).

*TNP-lation of Gly-*DL*-Val with ClTNB*—A mixture of Gly-DL-Val (100 mg.), ClTNB (500 mg. in 10 ml. ethanol) and sodium acetate, (sodium bicarbonate, disodium phosphate or disodium citrate; 500 mg. in 5 ml. water), was shaken for five hours in dark place at room temperature. The mixture, after washing with benzene (twice, 20 ml. each) to remove ClTNB, was acidified (pH 1) and extracted with ether (thrice, 25 ml. each). From the residues of the combined ether-extracts, containing TNP-Gly-DL-Val and picric acid, the former was recrystallized from hydrated methanol, as yellow prism. m.p. 206°–207.5° (decomp.) Yield 180 mg. (81%)

By means of paper chromatographic analysis of the reaction mixture, about 45, 65 and 85 per cent of the peptide were estimated to be TNP-lated after 30, 60 and 90 minutes, respectively. The amount of picric acid was increased by prolonging the reaction time, thus 1/25, 1/10 and 1 mole per mole of TNP-peptide, were formed after 1/2, 1 and 10 hours, respectively.

The TNP-lation in strong alkaline solution (pH 11 to 13) as reported by Hirayama (*14*) or the reaction at an elevated temperature (60° to 85°) as used for the DNP-lation with ClDNB (*27*) or with DNBS (*11*), were accompanied with undesirable decomposition of ClTNB. Using the method described in the above example, TNP-Gly, -L-Ala, -L-Pro, -Gly-Gly, -Gly-DL-Ser and -DL-Ala-DL-AspNH_2 could be also obtained in good yield, respectively. In the case of Lys, the α,ε-bis-substitution occurred, while ε-mono-TNP-Lys was obtained by the TNP-lation of copper derivative of Lys in which the α-amino group was blocked as a copper-complex (*28*).

Synthesis of ε-mono-TNP-L-Lys—To a suspension of cupri carbonate (100 mg.) in L-Lys solution (200 mg. of the mono-hydrochloride in 17 ml. water), which had been previously heated at 60° for 20 minutes to complete the cupri-complex formation, were added ClTNB (800 mg. in 14 ml. ethanol) and sodium bicarbonate (1 g.). After standing over night at room temperature, the precipitated TNP-L-Lys-cupri complex was gathered by the filtlation. The precipitates, after successive washing with water, ethanol and ether, was decomposed with hydrogen sulfide as a suspension in *N* hydrochloric acid. The combined filtrate and washings of the cupri sulfide, were treated with charcoal, washed with benzene, then concentrated. The separated ε-mono-TNP-L-Lys was recrystallized as the monohydrochloride monohydrate from *N* hydrochloric acid. m.p. 198°-199°. Yield 235 mg. (54%)

In the case of TNP reaction mixture from His or Tyr, not only the expected three spots due to α-mono-TNP-amino acid, picric acid and unreacted ClTNB, but also another yellow spot were detected on the paper chromatograms. The fourth spot showed ultraviolet spectrum, characteristic to TNP-amino compounds but no Pauli's diazo-reaction, and their R_f values were similar to those of bis-DNP-derivatives. The amounts were increased by prolonging the reaction time in the presence of excess amount of ClTNB*. In the case of TNP-lation of Arg in strong alkaline solution at pH 13, too, such an additional TNP derivative could be detected. These facts indicate that guanidino group of Arg in strong alkaline solution, imidazole imino group of His and phenolic hydroxy group of Tyr, were also TNP-lated with ClTNB, but with much less velocity than amino group was done. These results were similar to the case of the DNP-lation with FDNB (*24*, *25*, *26*).

II. TNP-lation with TNBS—The TNP group of the sulfonic acid coupled with amino group in slight alkaline solution at pH 7.5 to 8.5 and at room temperature almost quantitatively, as shown in the following example.

TNP-lation of Gly with TNBS—Gly (75 mg.), TNBS (200 mg.) and sodium bicarbonate (200 mg.) were dissolved in water (10 ml.) and the solution was kept in dark place at room temperature for two hours. The solution colored yellow almost immediately and the intensity increased during the initial one hour. On the acidification of resulting solution to pH 1, TNP-Gly crystallized out almost quantitatively (215 mg.) without any contamination of picric acid. m.p. 158°–160° (161° after once recrystallization from hydrated methanol)

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* These fourth spots were also observed on the further treatment of the corresponding α-mono-TNP-compounds with ClTNB.

The identity of the product was confirmed by the elememtary analysis, by no depression of the melting point on admixture with authenic TNP-Gly obtained with ClTNB (*14*) and by the comparison of some properties (R_f, ultraviolet and infrared absorption spectra) with those of a genuine specimen.

Using the similar method, crystalline α-mono-TNP-L-Ala, -L-Val, -L-Leu, -L-Ser, -L-Thr, -L-Asp, -L-Glu, -L-Met, L-Tyr, -Gly-Gly, -Gly-DL-Val, -Gly-DL-Ser, -DL-Ala-DL-AspNH$_2$ and -L-Leu-L-Tyr were obtained in more than 80 per cent yield, respectively. α-Mono-TNP-L-His and -L-Arg were also prepared according to this method, except the both amphoteric products could be crystallized out at pH 4, instead of pH 1.

Those products, which were new compounds except TNP-Ala and -Val, were concluded to be α-mono-TNP-derivatives by their elementary analyses, by their ultraviolet absorption spectra and from an analogy to the reaction of TNBS with Gly under the same conditions. The mono-TNP-Tyr and -Leu-Tyr, different from the N, O-bis-substituted derivatives, were Millon- and diazo-reactions positive and their color intensities were comparable to those of equivalent free Tyr. The presence of free phenolic hydroxy group in TNP-Leu-Tyr was also confirmed by the identification of TNP-Leu and free Tyr but no picric acid in the acid hydrolysate (6 *N* hydrochloric acid, 5 hours at 110°). α-Mono-TNP-L-His was Pauli's diazo- and α-mono-TNP-L-Arg was Sakaguchi-reactions positive, respectively. The both color intensities were as the same as those of equivalent free His (*29*) and Arg (*21*), respectively.

Higher peptides, oxidized insulin A and B, could be also TNP-lated under the similar conditions, giving water soluble TNP derivatives. TNP-oxidized insulin A had a molar extinction coefficient corresponding to mono-TNP derivative, while B gave a product corresponding to bis-TNP derivative.

TNP-lation of Oxidized Insulin A—Oxidized insulin A (30 mg.), TNBS (100 mg.) and sodium bicarbonate (100 mg.) were dissolved in 10 ml. water and the solution was kept for five hours in dark place at room temperature. The resulting orange-colored solution was added to a column of talc-Celite (1 : 1 *w/w*), 1 × 10 cm., which had been well washed with *N* ammonia. TNP-oxidized insulin A adsorbed on the top of the column as a narrow band, was washed thoroughly with water (300 ml.) and eluted with a mixture of one per cent ammonia and methanol (1 : 1 *v/v*)*. By the concentration of eluate in a flash evaporator, followed by the lyophilisation, TNP-oxidized insulin A could be obtained as amber-colored powder. Yield 28 mg. λ_{max} 338 mμ., ε 1.11×10^4 (4% NaHCO$_3$).

Most remarkable facts in the TNP-lation with TNBS were that no substitution in imino groups (of His and Pro), nor in hydroxy groups (of Tyr,

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* For the elution of bis-TNP derivatives, a mixture of one per cent ammonia and methanol (1 : 2*v/v*) should be used (30).

Ser, Thr, water and alcohol) could be detected, even at elevated temperature (45° to 85°) or after a prolonged reaction time (up to 3 days) in the presence of a great excess of TNBS (up to 20 times molar), indicating the TNP group coupled with only primary amino group, selectively.

III. Some Properties of TNP-Amino Acids and -Peptides—In general, the

TABLE

Preparation and Physical Constants

TNP	Preparation		m.p.	ε
	a	b		c
Glycine	ClTNB TNBS	MeOH-W	161°	1.54×10^4 (348 mμ.)
L-Alanine	ClTNB TNBS	6*N* HCl	90.5°	—
L-Valine	TNBS	MeOH-W	95°	1.44 ,, (350 ,,)
L-Leucine	TNBS	MeOH-W	98°-99°	1.66 ,, (350 ,,)
L-Serine	TNBS	MeOH-W	185°-188°	1.50 ,, (350 ,,)
L-Threonine	TNBS	MeOH-W	167°-168°	1.59 ,, (350 ,,)
L-Aspartic acid monohydrate	TNBS	NaOH→HCl pH 1	151°-152°	1.34 ,, (350 ,,)
L-Glutamic acid	TNBS	MeOH-W	165°-166°	1.50 ,, (350 ,,)
L-Methionine	TNBS	6 *N* HCl	125°-126°	—
L-Arginine	TNBS	NaOH→HCl pH 4	195°	1.52 ,, (350 ,,)
L-Histidine monohydrate	TNBS	NaOH→HCl pH 4	175°	1.52 ,, (350 ,,)
L-Tyrosine	TNBS	MeOH-HCl	168°	—
L-Proline	ClTNB	MeOH-W	164°-165°	—
L-Lysine (ε-mono) monohydrochloride monohydrate	ClTNB	*N* HCl	198°-199°	1.45 ,, (346 ,,)
L-Lysine (α, ε-bis)	ClTNB TNBS	MeOH-heptane	—	2.62 ,, (350 ,,)
Glycylglycine	ClTNB TNBS	MeOH-W	221°-222°	1.32 ,, (344 ,,)
Glycyl-DL-valine	ClTNB TNBS	AcOEt	206°-207°	1.23 ,, (342 ,,)
Glycyl-DL-serine	ClTNB TNBS	AcOEt-heptane	210°-211°	1.26 ,, (348 ,,)
L-Leucyl-L-tyrosine	TNBS	MeOH-W	127°-128°	1.29 ,, (350 ,,)
DL-Alanyl-DL-aspargine	ClTNB TNBS	MeOH-W	199°	1.20 ,, (350 ,,)

(a) TNP reagent (b) Solvent for recrystallization. W, water; MeOH, methanol;
HCl (c) In 4% $NaHCO_3$ (d) In *N* HCl

properties of TNP-amino acids and -peptides were similar to the corresponding DNP derivatives. As already mentioned, the purification of TNP compounds could be achieved, according to the methods for that of the DNP derivatives (*2*, *3*, *31*), although some of the formers could be crystallized with more difficulties.

I

of TNP-Amino Acids and -Peptides

(λ_{max})	Elementary analysis					
d	Found			Calc.		
	C	H	N	C	H	N
1.25×10^4 (340 mμ.)	33.1	2.36	19.1	33.5	2.10	19.6
1.27 ,, (340 ,,)	35.8	2.91	19.0	36.0	2.69	18.7
1.34 ,, (340 ,,)	40.0	3.53	17.0	40.2	3.69	17.1
1.48 ,, (340 ,,)	42.3	4.32	16.9	42.1	4.12	16.4
1.42 ,, (340 ,,)	34.4	2.38	17.4	34.2	2.56	17.7
1.33 ,, (340 ,,)	35.7	3.77	16.7	36.4	3.06	17.0
1.15 ,, (340 ,,)	33.2	2.95	15.7	33.4	2.79	15.7
1.40 ,, (340 ,,)	36.6	2.48	15.3	36.9	2.81	15.4
1.34 ,, (340 ,,)	36.6	3.45	15.8	36.7	3.36	15.6
—	37.1	4.12	24.9	37.4	3.92	25.5
1.35 ,, (340 ,,)	37.5	3.14	21.9	37.5	3.29	22.1
1.38 ,, (340 ,,)	—	—	14.3	—	—	14.3
1.29 ,, (362 ,,)	40.1	2.98	17.0	40.5	3.07	17.2
1.33 ,, (345 ,,)	35.1	3.69	17.7	35.1	3.90	17.1
—	—	—	—	—	—	—
1.10 ,, (338 ,,)	35.7	2.41	19.8	35.0	2.63	20.4
1.09 ,, (338 ,,)	41.2	4.05	17.5	40.7	3.91	18.2
1.10 ,, (340 ,,)	—	—	18.8	—	—	18.9
1.09 ,, (340 ,,)	89.1	4.28	14.2	40.3	4.56	13.8
1.05 ,, (339 ,,)	—	—	20.5	—	—	19.9

AcOEt, ethyl acetate; NaOH→HCl, dissolved in diluted NaOH and acidified with

Melting point and molar extinction coefficient of TNP-amino acids and -peptides are listed in Table I, together with their preparation method and with the results on their elementary analysis.

Ultraviolet absorption spectra of TNP compounds were rather similar to

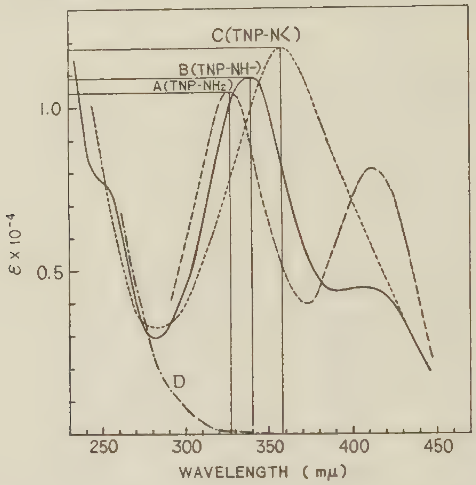

Fig. 1. Ultraviolet absorption spectra of various TNP-compounds in *N* HCl.
(A) Picramide, (B) TNP-Leu-Tyr, (C) TND-Pro-Gly, (D) TNBS

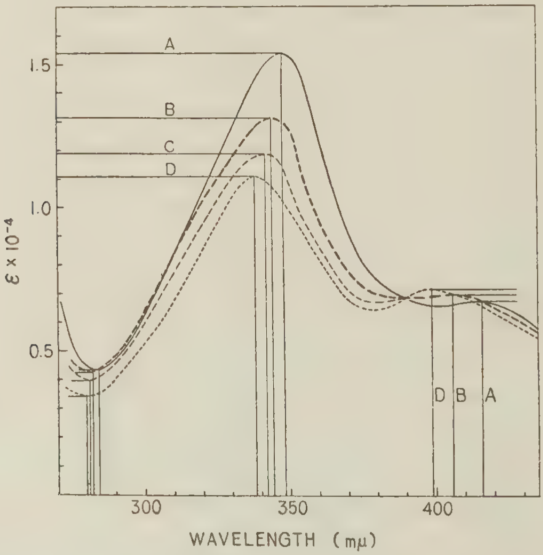

Fig. 2. Ultraviolet absorption spectra of TNP-amino acid and -peptides in 4% $NaHCO_3$.
(A) α-mono-TNP-L-His, (B) TNP-Gly-Gly, (C) TNP-Gly-Gly-Ala, (D) TNP-oxidized insulin A

those of the corresponding DNP derivatives, especialy in the relations among those of picramide (TNP-NH_2), TNP-amino acids (TNP-NHR) and TNP-

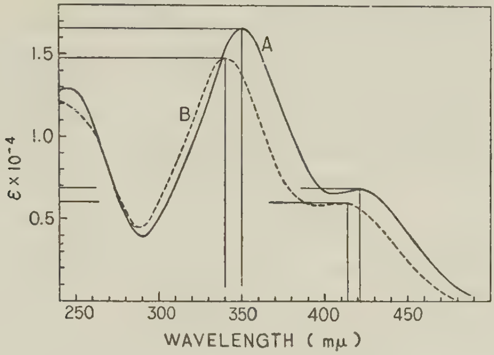

Fig. 3. Ultraviolet absorption spectra of TNP-leucine in acidic and alkaline solutions.
(A) in 4% sodium bicarbonate, (B) in *N* hydrochloric acid

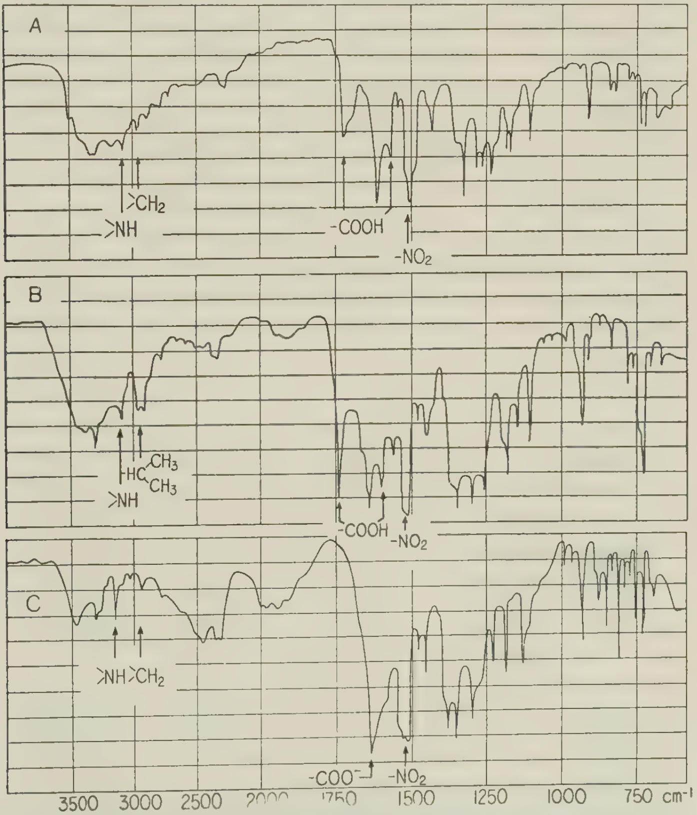

Fig. 4. Infrared spectra of TNP-amino acids in potassium bromide.
(A) TNP-L-Tyr, (B) TNP-L-Val, (C) α-mono-TNP-L-His

imino acid (TNP-NRR′), as shown in Fig. 1, between those of TNP-amino acids and -peptides, as shown in Fig. 2, and between those in acidic and alkaline solutions, as shown in Fig. 3. In the case of DNP derivatives, these relations had been studied in details (*2*, *3*, *32*). Compared with DNP compounds, the corresponding TNP derivatives had a slight (about 15 per cent) less molar extinction coefficient ($1.2\text{–}1.6 \times 10^4$) at the absorption maximum in a region of a little (about 15 mμ.) shorter wavelength (340–350 mμ.) and had a larger shoulder (or a small second peak) at a region from 400 to 420 mμ., which was especially remarkable in the case of picramide, as shown in Fig. 1.

Infrared absorption spectra of typical TNP-amino acids are shown in Fig. 4. Besides many absorption bands characteristic to each amino acid,

Table II

R_f Values of TNP-Amino Acids

TNP-	R_f		
	1	2	3
Glycine	0.39	0.46	—
Alanine	50	52	—
Valine	56	61	—
Leucine	46	63	—
Isoleucine	45	62	—
Phenylalanine	38	64	—
Tyrosine (α-mone-)	40	53	—
(N, O-bis-)	12	65	—
Methionine	50	60	—
Aspartic acid	66	03	—
Glutamic acid	61	05	—
Cysteic acid	69	02	0.22
Proline	62	74	—
Tryptophan	20	tailing	—
Arginine (α-mono-)	57	37	0.65
(N, N′-bis-)	09	71	—
Histidine (α-mono-)	65	34	0.90
(N^{α}, N^{1}-bis-)	08	63	—
Lysine (ε-mono-)	67	30	0.67
(α, ε-bis-)	07	58	—
Picric acid	33	59	0.68
Picramide	00	78	—
TNBS	51	—	0.47
ClTNB	00	—	—

Developed with (1) 1.5 M phosphate buffer (pH 5.4), (2) *n*-butanol saturated with 5% ammonia and (3) *n*-butanol-acetic acid-water (4 : 1 : 2 *v*/*v*).

two strong bands due to the nitro (1510–1540 cm.$^{-1}$) and to unionized carboxyl (about 1700 cm.$^{-1}$) groups were notable in common, except α-mono-TNP-Arg and -His. The absence of the band at 1700 cm.$^{-1}$ and the presence of an intense band at 1650 cm.$^{-1}$ in the latters may indicate that these amphoteric derivatives present as the betain structure in crystal, differing from the usual TNP-amino acids.

R_f values of TNP-amino acids, developed on Toyo Filter Paper No. 51, using *n*-butanol-ammonia and 1.5 *M* phosphate buffer as the solvents, are summarized in Table II. Two dimensional method, using the former as the first solvent and the latter as the second, could separate all ether-soluble TNP-amino acids (1 to 50 μg. each), except the leucine isomers, on a single piece of paper (60×60 cm.). The paper chromatograms, on which the intense color of TNP-amino acids allowed them to be located very easily, was rather similar to those of DNP-amino acids separated by the same solvent system (*13*, *19*). For the separation of ether-insoluble TNP-amino acids, α-mono-TNP-CySO$_3$H, -Arg, -His and ε-mono-TNP-Lys, a mixture of *n*-butanol, acetic acid and water (4 : 1 : 2 *v/v*) gave the best results.

Photo-stability. Most important in TNP-amino acids and -peptides was the photodecomposition, which was much more than that of DNP-amino acids (*2*, *3*, *33*). Only a slight exposure of the paper chromatogram to sunlight, an appreciable photodecomposition occurred, which resulted into a color change from yellow to brown and into a tailing of spots. In the aqueous solution, too, they decomposed rapidly and picramide was presumed to be an intermediate of the photoreaction, by means of ultraviolet spectral and paper chromatographic analyses (*34*).

Acid stability of TNP-amino acids in 6 *N* hydrochloric acid at 110°, was also inferior to that of the corresponding DNP-amino acid (*2*, *33*), but only slightly. Thus, usual TNP-amino acids (TNP-Ala, -Val, -Leu, -Ser, -Thr, -Asp, -Glu and -Arg) were recovered in 55 to 65 per cent yield, after heating the solution at a concentration of 10 μg. per ml., in 6 *N* hydrochloric acid for 5 hours at 110°. α-mono-TNP-His was also recovered in a similar yield, differing from acid-labile N^1-DNP- and -TNP derivatives. The recovery of ε-mono-TNP-Lys was fairy good (85–95 per cent), while that of TNP-Gly was far less (30–35 per cent) and TNP-Pro was decomposed completely, giving picric acid (70 per cent) and free Pro. In the case of TNP-Gly-Gly, -Gly-Ser, -Leu-Tyr and -Ala-AspNH$_2$, too, TNP-Gly, -Leu and -Ala could be recovered in 34, 30, 56 and 60 per cent yields under the above-mentioned conditions respectively.

DISCUSSION

It may not be so surprising that the TNP group of TNBS or ClTNB was introduced into amino group with faster velocity under far milder conditions, than the DNP group of DNBS (*9*, *10*, *11*) or ClDNB (*27*) did; as the formers had an additional ortho-nitro group, which cause further to reduce the electron density of sulfonic acid or chlorine and to accelerate the removal from aromatic ring. There have been several reports on the similar desulfonative condensation of aromatic sulfonic acid with amino compounds (*35*, *36*), but the precise mechanism seems to be still unclear. According to Eisen *et al.* (*10*), the desulfonative DNP-lation with DNBS occured only in the presence of oxygen, suggesting the oxidative cleavage of DNP-SO_3H bond resulted into DNP-NHR and inorganic sulfate. In the case of TNBS, on the other hand, no oxygen uptake was observed during the coupling with amino acids in Warburg manometer. The details are now being studied in this laboratory, together, with the other types of nitrophenylation of amino group under the removal aromatic nitro (*12*, *13*, *15*, *16*), amino (*13*, *16*) or alkoxy (*13*, *16*) groups.

The most striking fact in the desulfonative TNP-lation with TNBS, is that the TNP group coupled with primary amino group, selectively. Ikenaka (*9*) has already reported that DNBS reacted with Taka-amylase in water solution at pH 10 to 11 rather slowly, giving ε-mono-DNP-Lys but no imidazole imino-DNP-His nor O-DNP-Tyr. One of the present authors had also got the similar conclusions, using known amino acids and hemoglobin (*37*). TNBS reacted with amino group of protein in water solution at pH 8 and at room temperature, rapidly but only partially, presumably for the steric reasons (*13*). So it should possess more advantages for partial labeling of protein in connection with the biological essentiality of amino group or with the analysis of the masked amino group.

The TNP-lation of amino acids and lower peptides with TNBS occurred quantitatively within two hours, without any formation of undesirable products such as picric acid. Each resulting TNP derivative had a high molar extinction coefficient (1.2 to 1.6×10^4 at 350 mμ.) in a similar magnitude, and TNBS itself had no appreciable light absorption in a range of longer wavelength than 300 mμ.. These facts suggest a possibility of a convenient spectrophotometry of amino acids and lower peptides, after the TNP-lation with TNBS. The details will be reported soon in this journal (see also *29*, *30*).

TNP-amino acids were generally less stable than the corresponding DNP-amino acids. However, terminal amino acids revelated from peptides or proteins by the action of carboxy- or amino-peptidases, and amino alcohol derived from the reduced C-terminal of protein (*2*, *3*, *11*) may be easily identified and assayed as the TNP derivatives. It is because that the reagent gave no undesirable by-products such as 2,4-dinitrophenol from FDNB and that the excess amount of ether-insoluble TNBS can be easily separated by the ether extraction of the desired TNP compounds. Especially, in the case of His peptides, TNP-lation followed by the acid hydrolysis, seems to be promising, as the *N*- and non-terminal His, which gives acid-labile N^{α},N^1-bis- and N^1-mono-DNP-His in the case of the usual FDNB method, could be identified and assayed as the acid stable α-mono-TNP- and free-His, respectively (*29*).

SUMMARY

1. Amino acids and lower peptides could be TNP-lated both with ClTNB and with TNBS.

2. TNBS coupled with primary amino group but not with water nor other groups commonly encountered in protein, in water solution at pH 8 and at room temperature.

3. The preparation and some properties such as m.p., R_f, molar extinction coefficient, photo- and acid-stabilities, of TNP-amino acids and -peptides (α-mono-TNP-Gly, -L-Ala, -L-Val, -L-Leu, -L-Tyr, -L-Arg, -L-Asp, -L-Glu, L-Met, -L-His, ε-mono- and α,ε-Bis-TNP-Lys; α-mono-TNP-Gly-Gly, -Gly-DL-Val, Gly-DL-Ser, -DL-Ala-DL-AspNH_2 and -L-Leu-L-Tyr) are reported, together with the ultraviolet and infrared absorption spectra of some typical TNP derivatives.

4. These results were briefly discussed with connection of possible application of TNBS in protein chemistry.

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STUDIES ON ADENOSINE TRIPHOSPHATE-PHOSPHATE EXCHANGE REACTION

I. THE ADENOSINE TRIPHOSPHATE-PHOSPHATE EXCHANGE REACTION DURING OXIDATIVE PHOSPHORYLATION

By KOZO IWASA

(*From the Department of Biology, Faculty of Science, University of Osaka, Osaka*)

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Cohn (*1*) found that under conditions which presumably could not support electron transport, mitochondria catalyze the exchange reaction of ATP* and *i*P. Later this reaction was studied by Swanson (*2*) and Boyer *et al.* (*3*) who found that the reaction was closely related to oxidative phosphorylation.

In 1957, Cooper and Lehninger (*4*) also observed, with a digitonin preparation (submitochondria) that the exchange reaction is inhibited by inhibitors of oxidative phosphorylation but not by inhibitors of oxidation. Therefore, it was reasonably concluded by all these investigator that the ATP-*i*P exchange reaction is the manifestation of the reversal of some reaction step in oxidative phosphorylation.

Recently, Wadkins and Lehninger (*5*), and Bronk and Kielley (*6*) reported that the exchange reaction varies with the oxidized (*5*) or reduced (*6*) state of respiratory carriers, and suggested that a primary high energy complex is formed with phosphate and some of the carriers.

This paper reports that the ATP-*i*P exchange reaction during oxidative phosphorylation is suppressed as compared with that of a reaction which does not involve electron transfer.

MATERIALS AND METHODS

Mitochondria were prepared from the liver of albino rats according to the method of Schneider and Hoogeboom (*7*) using 0.3 *M* sucrose. They were centrifuged down between $700\times g$ and $5{,}200\times g$ for five minutes.

Esterified phosphate was determined according to the method of Cooper and Lehninger (*5*) using radioactive iP^{32}. Radioactivity was measured using a thin-mica end-window Geiger-Müller tube. *i*P was measured by the method of Fiske and Subbarow (*8*). Oxygen uptake was measured in a Warburg manometer using trimethylpentane instead of Brodie solution (*9*). Adenine nucleotides were separated and measured by the method of Cohn and Carter (*10*). Purified ATP (crystalline) and ADP were prepared as described previously (*11*).

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* The following abbreviations are used in this paper: ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; *i*P, inorganic orthophosphate; EDTA, ethylenediaminetetraacetate.

RESULTS

Inorganic P decrease was measured in an oxidative phosphorylation system containing succinate as substrate and a hexokinase-glucose system as a trapper for the ATP generated. Table I, column 5 shows the net decrease in *i*P, which is the difference between the residual *i*P in the control and in the test mixture. This is always less than the iP^{32} incorporated into organic compounds, as calculated from the radioactivity in the water layer after extraction of *i*P by isobutanol-benzene, Table I, column 3. Column 4 shows the net amount of iP^{32} incorporated, as determined by substracting the *i*P of the control from that of test reaction mixture. This is always less than the decrease in *i*P. Therefore these are probably two ways in which iP^{32} can be incorporated (iP^{32} incorporated). These may be firstly, P-esterification coupled with oxidation, which is estimated by decrease in *i*P and secondary iP^{32}-incorporation by the ATP-*i*P exchange reaction. The latter is affected by the *i*P concentration and decreases as oxidative phosphorylation proceded. Representative data of these reactions are shown in Table I.

TABLE I

ATP-iP Exchange Reaction During Oxidative Phosphorylation and Effect of Thermal Ageing on the Reaction

Warburg vessels contained: Main chamber; 0.1 ml. of 0.1 *M* iP^{32}, 0.1 ml. of 0.1 *M* KF, 0.1 ml. of 0.05 *M* $MgCl_2$, 0.1 ml. of 0.01 *M* EDTA, 0.1 ml. of 0.05 *M* ATP (5 μmoles), 0.1 ml. of 0.2 *M* glucose containing hexokinase (capable of reacting with 2μmoles of ATP for 10 minutes under these conditions), and 0.1 ml. of 0.2 *M* succinate. Side arm; 0.1 ml. of mitochondrial suspension in 0.3 *M* sucrose (equivalent to 100 mg. fresh liver). Centre well; 0.1 ml. of 20% KOH. Total volume, 1.0 ml. pH 7.2. Temperature, 25°. After 20 min. incubation, the reaction was stopped with 40% trichloroacetic acid. The mixture was centrifuged at low speed and the *i*P and esterified iP^{32} measured as described in the text.

Preincubation period (min.)	Succinate added	O_2 consumed (μatoms)		iP^{32} incorporated (μmoles)		*i*P decrease (μmoles)	P/O ratio		
		Found (1)	Net (2)	Found (3)	Net (4)	Net (5)	(5)/(2)	(3)/(1)	(4)/(2)
5	+	2.17	2.17	3.81	1.90	3.10	1.43	1.76	0.88
5	−	0.00		1.91				—	
15	+	1.88	1.78	3.32	1.24	2.80	1.49	1.77	0.70
15	−	0.10		2.08				—	
25	+	2.74	2.63	2.72	0.84	1.61	0.61	0.99	0.32
25	−	0.11		1.88				—	

Mitochondrial suspension are preincubated with 0.3 *M* sucrose in the side arm of a manometric vessel for 5, 15 and 25 minutes at 25°. Oxidative phosphorylation was thereby decreased by thermal ageing. The decrease in *i*P was greater than *i*P^{32} incorporation, as shown in the Table I, columns 4 and 5. This could be explained by assuming that *i*P^{32} incorporation involves an exchange reaction and is inhibited during oxidative phosphorylation. Therefore the value for the exchange reaction may be calculated by subtracting column 5 from column 3 in Table I.

When the hexokinase-glucose system was omitted and the reaction mixture was not shaken the effect of ageing was magnified. The exchange reaction was diminished by liberation of *i*P^{32} from ATP, owing to the activity of ATP-ase. In an oxidative phosphorylation system containing the hexokinase-glucose system, ATP is broken down to ADP. It is therefore possible to assume that when the mitochondrial adenylate kinase is active ADP can react with *i*P. To examine the ADP-*i*P exchange reaction, a system was studied in which ATP was replaced by ADP. As shown in Fig. 1, there is definitely an ADP-*i*P exchange reaction, but it is week as compared with the ATP-*i*P exchange reaction. There is much *i*P liberation in the ATP-*i*P reaction system without added oxidizable substrates. It is conceivable that the liberation of *i*P is dependent on the activity of the mitochondrial ATP-ase (*12*). In the ADP-*i*P exchange reaction, it is reasonable to assume that ATP is formed by the action of mitochondrial adenylate kinase and then there is exchange between ATP and *i*P. Adenylate kinase from animals is known to be partially inhibited by fluoride (*13*) and EDTA (*14*). Fig. 2 shows that the exchange reaction, contrary to expectation, is stimulated by fluoride.

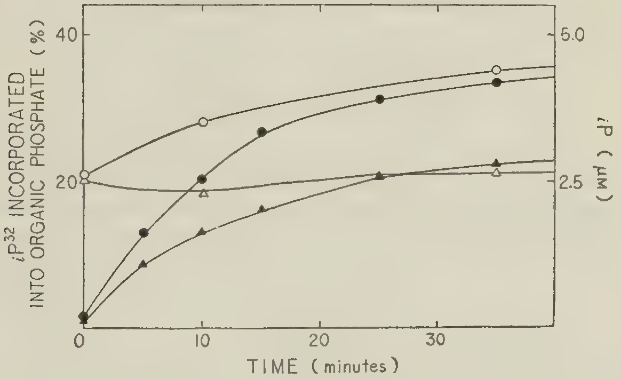

FIG. 1. The exchange reaction and liberation of *i*P by ATP and ADP. Reaction mixture contained 0.1 ml. of 0.06 M *i*P^{32} (ca. 2×10^4 c.p.m.), 0.1 ml. of 0.01 M EDTA, 0.1 ml. of 0.05 M $MgCl_2$, 0.2 ml. of 0.04 M ATP (or 0.2 ml. of 0.02 M ADP), 0.1 ml. of mitochondrial suspension, water to 1.0 ml. pH 7.2 Temperature 25°.

—●—; Exchange with ATP —▲—; Exchange with ADP

—○—; Liberation of *i*P from ATP. —△—; Liberation of *i*P from ADP.

Probably the ATP-ase which suppresses the exchange reaction is inhibited

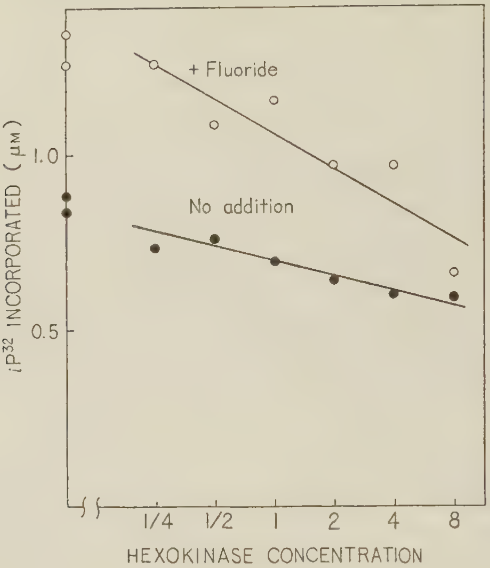

FIG. 2. Effect of hexokinase and fluoride on *i*P-incorporation. Concentration of hexokinase containing glucose is expressed as a relative amount. A concentration of 1.0 represents the amount able to react with 2 μmoles of ATP for 10 minutes under these conditions. 10 μmoles flouride added per ml. of reaction mixture.

TABLE II

Effect of EDTA on the ATP-iP Exchange Reaction

The reaction mixture contained 0.1 ml. of 0.1 *M* KF, 0.1 ml. of 0.05 *M* $MgCl_2$, 0.1 ml. of 0.2 *M* glucose containing hexokinase, 0.1 ml. of mitochondrial suspension, EDTA as indicated. The reaction was started with addition of 0.1 ml. of 0.1 *M* iP^{32} and 0.1 ml. of 0.05 *M* ATP. pH, 7.2. Temperature, 27°. Reaction period, 10 min. without shake.

EDTA added (μmoles)	Exchange activity (iP^{32} incorporation) (μmoles)
0	0.14
1	0.47
2	0.48
4	0.73
10	0.28
2*	1.55

* Without addition of glucose-hexokinase.

in the presence of fluoride. Addition of concentrated hexokinase also inhibited the exchange activity, while EDTA inhibited it slightly at high concentration but activated it at low concentration (10^{-5} *M*) as shown in Table II. Mitochondrial adenylate kinase possibly has different properties from myosin. An incubation as in Fig. 1 was made with either ATP or ADP and the $i\text{P}^{32}$ incorporated into adenine nucleotide was assayed chromatographically. The reaction was stopped by addition of 0.5 *N* perchloric acid. The mixture was centrifuged at low speed and neutralized with potassium hydroxide. After cooling, the perchlorate was removed. The extract was charged on a column of anion exchange resin (Dowex 1, ×9). It was eluted with a mixture of hydrochloric acid and sodium chloride and ATP, ADP and AMP being separated by the method of Cohn and Carter. Adenine nucleotides

TABLE III

Chromatographic Assay of Adenine Nucleotide in the Exchange Reaction System with ATP or ADP as Substrate

The reaction systems as in Fig. 1. Assay procedure was as described in the text.

		ATP	ADP	AMP
ATP added (8 μmoles)	mμmoles	7,265	614	6
	Relative count	8,050	58	0
ADP added (4 μmoles)	mμmoles	961	998	1,108
	Relative count	1,062	88	0

TABLE IV

Effect of Various Compounds on the ATP-iP Exchange Reaction

Experimental conditions as in Table II.

Materials added (Final concentration)	Exchange activity ($i\text{P}^{32}$ incorporation, μmoles)	Per cent of control
None	1.55	100
Sucrose (0.4 *M*)	0.17	11
2,4-Dinitrophenol (10^{-4} *M*)	0.17	11
,, (10^{-5} *M*)	1.22	79
Ascorbate (10^{-2} *M*)	1.54	99
Ferricyanide (10^{-4} *M*)	1.61	104
Ferrocyanide (10^{-4} *M*)	1.67	108
Arsenate (10^{-2} *M*)	1.07	69
ADP in place of ATP	1.02	100
ADP plus AMP (16×10^{-3} *M*)	0.92	92

were determined spectroscopically and the radioactivity measured in each fraction of the eluate. The data shown in Table III, suggest the ADP-*i*P exchange reaction is a pretended reaction which is mixed with adenylate kinase.

Sucrose (*2*, *4*), 2,4-dinitrophenol (*2*, *4*), arsenite (*4*), and arsenate (*2*) are known to inhibit the exchange reaction. Wadkins and Lehninger (*5*) reported that in their oxidized state, electron carriers greatly stimulate the exchange reaction of digitonin particles. Conversely Bronk and Kielley (*6*), observed that the rate of exchange is greatly increased by first reducing the components of the electron transport chain in a preparation of mitochondria prepared by supersonic vibration. To confirm these effects, the various compounds shown in Table IV, were added to the reaction mixture.

Sucrose and 2,4-dinitrophenol strongly inhibited the exchange reaction. Arsenate inhibited the reaction slightly. When AMP was added, ADP was used in place of ATP, AMP, was also found to inhibit the reaction slightly. Ascorbate, ferrocyanide and ferricyanide were without effect on the exchange reaction. The absence of effect of these substances is discussed in the following report.

DISCUSSION

Phosphorylation coupled with oxidation is associated with particles, whether mitochondrial or the fragmental particles. The activity is often lost during preparation, since the electron carriers dissociate from the particles. Therefore the mechanism of oxidative phosphorylation is not yet clear, although there is much information available about it (*15*, *16*).

It seems probable that the reaction steps of oxidative phosphorylation, namely the formation of ATP from ADP and *i*P, are coupled with the passage of electrons *via* the cytochromes and related enzymes. Probably there are three reactions involved as follows; (i) activation of some materials by oxidation of the electron carriers, (ii) trapping of the energy as phosphate bonds in some materials, (iii) ATP formation from ADP. Whether the enzymes of each steps can be separated from the particles or whether an enzyme protein plays a part in the reactions, will not be discussed here. The ATP-*i*P exchange reaction corresponds to a reversal of reactions (ii) and (iii).

Therefore during oxidative phosphorylation, the ATP-*i*P exchange reaction must occur in parallel with phosphorylation. Even when for oxidative phosphorylation, ADP was usually used as an acceptor of *i*P and a hexokinase-glucose system as a trapper of ATP, there was much ATP-*i*P exchange, (Table I and Fig. 1) owing to the constant formation of ATP from ADP by the adenylate kinase of intact mitochondria (Table III). This was confirmed by chromatography of the adenine nucleotide from a reaction mixture containing ATP or ADP as exchangable substrates. The conclusion was also supported by the result that the exchange activity decreased inversely with the concentration of hexokinase. Moreover it was stimulated by fluoride, which probably inhibited mitochondrial ATP-ase.

Bowen and Kerwin reported that the adenylate kinase of muscle is inhibited by EDTA, therefore the inhibitory effect of EDTA on the ADP-*i*P exchange reaction was investigated. Table II shows that, contrary to expectation, EDTA at a low concentration stimulates the exchange, probably by protecting the mitochondria from ageing. Moreover the exchange reaction was slightly inhibited by AMP.

The exchange reaction is inhibited by specific inhibitors of oxidative phosphorylation (*4*). It is also inhibited by some materials which do not inhibited oxidative phosphorylation such as sucrose and ADP (*4*) probably owing to alternation in the balance of the reaction. Ferrocyanide, ascorbate and ferricyanide which change the oxidation-reduction state of the carriers, did not inhibit the exchange reaction. The relation of the exchange reaction to the oxidation state of the carriers will be discussed in the next report.

SUMMARY

1. The ATP-*i*P exchange reaction was studied in mitochondria. The exchange reaction was observed in a system with ADP as *i*P-acceptor and a hexokinase-glucose system as ATP-trapper due to the adenylate kinase in the mitochondria.

2. With succinate as substrate the exchange reaction was suppressed during oxidative phosphorylation as compared with substrates which presumably did not support electron transport.

3. The exchange reaction was inhibited by some specific inhibitors of oxidative phosphorylation, but not ascorbate, ferrocyanide or ferricyanide.

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STUDIES ON THE PHYSIOLOGICAL CHEMISTRY OF THE SALIVARY GLANDS

XLIV. MOLECULAR WEIGHT OF SALIVA-PAROTIN-A

By YOSOJI ITO AND YUKIHO KUBOTA

(From the Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Tokyo)

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In previous communications (*1*, *2*), procedures have been described for the isolation of saliva-parotin-A from human mixed saliva. It has also been shown that saliva-parotin-A possesses remarkable activities of decreasing the blood serum calcium level and affecting the number of circulating leucocytes (*3*), and that it consists of fifteen kinds of amino acids among which aspartic acid, glutamic acid, glycine, proline and threonine are characteristic in their high contents (*4*). In the present work, we have determined the molecular weight of saliva-parotin-A by the methods of diffusion and ultracentrifugation. The value for the molecular weight calculated from the sedimentation and diffusion coefficients was 15,900, which was in good agreement with the value of 15,700 determined during the approach to sedimentation equilibrium.

EXPERIMENTALS

Material and Solution—Saliva-parotin-A was purified by the ethanol fractionation (*2*) from the crude preparation (the E-precipitate), which was extracted from human mixed saliva (*1*). The purified material was dissolved in a citrate buffer of ionic strength 0.2 and pH 5.8. Prior to every measurement, the protein solution was dialyzed against the same buffer for 20 hours at 0°.

Sedimentation—Both sedimentation and diffusion measurements were conducted in a Spinco Model E ultracentrifuge, and a 12 mm. synthetic boundary cell with a 4° sector was used throughout the experiments. In sedimentation measurements, the runs were carried out at a speed of 59,780 r.p.m. at room temperature (about 28°). The rotor temperature was measured by a rotor temperature indicator and control unit (RTIC) at each time of photographing. Four sedimentation measurements were made with protein concentrations of 0.2, 0.4, 0.6 and 0.8 g. per 100 ml. The distance from the boundary to the reference hole of the counterbalance was measured on the photographic plate by means of a microcomparator.

Diffusion—In diffusion measurements, the runs were carried out at a speed of 12,590 r.p.m., and the rotor temperature was maintained at 13.5° ± 0.1° by RTIC. At this low speed, the spreading of boundary due to centrifugal force was negligible because of the low sedimentation velocity of saliva-parotin-A. Four diffusion measurements were made with protein concentrations of 0.1, 0.2, 0.4 and 0.8 g. per 100 ml. Diffusion coefficients were computed by the maximum ordinate-area method from the tracings which were made from photographs at fivefold linear magnification with a photographic enlarger.

Measurement during the Approach to Sedimentation Equilibrium—The molecular weight determination during the approach to sedimentation equilibrium was made following the procedure of Ginsburg, Appel and Schachman (*5*) based on the original proposal of Archibald (*6*). The ultracentrifuge was run at a speed of 12,590 r.p.m., and the rotor temperature was maintained at 17.9°±0.1°. A conventional cell with a 4° sector was employed. The measurement was carried out with a protein concentration of 1 g. per 100 ml. Before filling the cell with the protein solution, 0.1 ml. of Dow-Corning No. 555 silicone oil was introduced into the cell, and 0.6 ml. of the protein solution was then added to it. Since the density of the oil is higher than that of the aqueous solution, it formed a thin transparent layer at the bottom of the cell during the centrifuge operation. The sharp interface thus formed provided a new base for the protein solution which had the outline of an arc drawn from the axis of revolution. This eliminated the convective disturbance which may have resulted if the solution was placed directly in the cell. Photographs were taken at 16 minutes intervals from the time 6 minutes after reaching the set speed.

The molecular weight, M_e, was calculated by the following equation (*5*),

$$M_e = \frac{RT}{(1-V\rho)\omega^2} \cdot \frac{\frac{1}{x_b}\left(\frac{dc}{dx}\right)_b - \frac{1}{x_m}\left(\frac{dc}{dx}\right)_m}{c_b - c_m}$$

where R is the gas constant, T is the absolute temperature, V is the partial specific volume, ρ is the density of the solution, ω is the angular velocity of the rotor in radians per second, c_m and $(dc/dx)_m$ are the concentration and concentration gradient at the position x_m, which is the distance from the axis of revolution to the meniscus. The corresponding quantities with the subscript b refer to the bottom of the cell. As the photographs were taken while the plateau region still remained, the term $(c_b - c_m)$ was calculated by the following equation (*5*),

$$c_b - c_m = \frac{1}{x_b^2}\int_X^{x_b} x^2 \frac{dc}{dx}\, dx + \frac{1}{x_m^2}\int_{x_m}^{X} x^2 \frac{dc}{dx}\, dx$$

where X is a position in the plateau region where dc/dx equals zero. x and dc/dx were evaluated from the enlarged tracing made from the photograph with an enlarger.

Partial Specific Volume—The partial specific volume was measured at two protein concentrations (0.8 and 1.2 g. per 100 ml.) at 20°±0.02° in a 2 ml. pycnometer of Ostwald type.

RESULTS

Sedimentation Coefficient—Representative ultracentrifugal patterns of salivaparotin-A are shown in Fig. 1. Plotting the reciprocal of $s_{20,w}$ against the protein concentration, C, a straight line is obtained as illustrated in Fig. 2. From the extrapolated value and the slope of the line, the variation of $1/s_{20,w}$ in relation to C may be represented by the following equation,

$$\frac{1}{s_{20,w}} = \frac{1}{1.51}(1+0.581C).$$

The value of $s_{20,w}$ at infinite dilution is thus determined to be 1.51 S. If $s_{20,w}$ is plotted against C, a curve, not a straight line, is obtained, and the identical value of $s_{20,w}$ at infinite dilution is obtained by extrapolating the curve to zero concentration.

Diffusion Coefficient—$D_{20,\ w}$ of saliva-parotin-A is in linear relationship with the protein concentration as shown in Fig. 3. As is seen, $D_{20,\ w}$ depends to some degree upon the concentration. The value of $D_{20,\ w}$ at infinite dilution

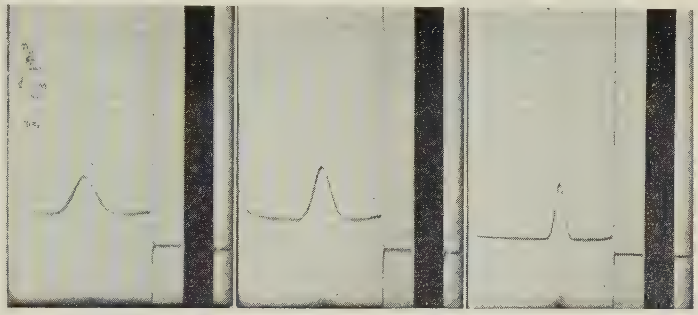

FIG. 1. Ultracentrifugal patterns of saliva-parotin-A in a citrate buffer of pH 5.8. Protein concentration, 0.8 g. per 100 ml.; rotor speed, 59,780 r.p.m. The numbers which follow the times represent the angles of the phase plate.

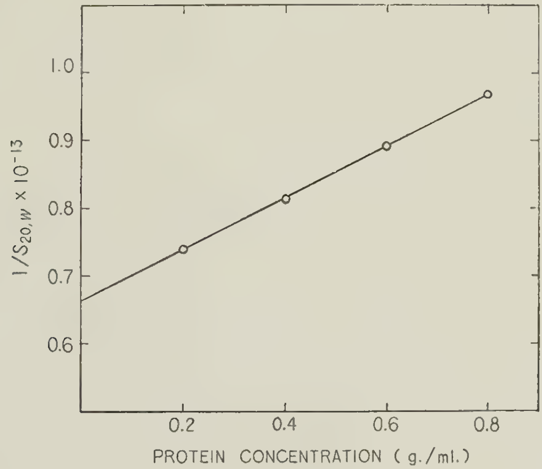

FIG. 2. Reciprocal plot of $s_{20,\ w}$ *versus* protein concentration for saliva-parotin-A measured in a citrate buffer of pH 5.8.

is thus obtained to be 7.41×10^{-7} cm.^{2} per second. The concentration dependence of $s_{20,\ w}$ and $D_{20,\ w}$ may be due to the mutual interaction among molecules of saliva-parotin-A.

Partial Specific Volume—The partial specific volume was computed to be 0.689 at 0.8 g. per 100 ml. and 0.686 at 1.2 g. per 100 ml. The mean value for the partial specific volume of saliva-parotin-A is thus 0.688 ml. per g.

Molecular Weight—The molecular weight, M_{sD}, and the frictional ratio were calculated from the values of $s_{20,w}$, $D_{20,w}$ and partial specific volume by the usual procedures, and were found to be 15,900 and 1.76, respectively.

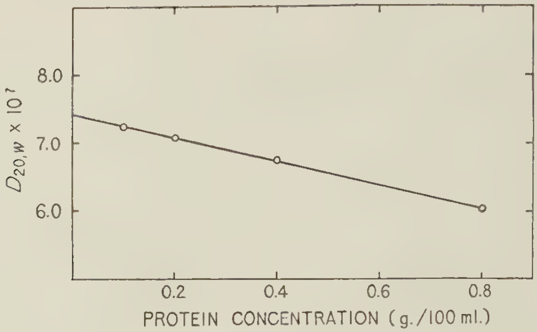

Fig. 3. Diffusion coefficient of saliva-parotin-A as a function of concentration in a citrate buffer of pH 5.8.

Representative ultracentrifugal patterns of saliva-parotin-A obtained during the approach to sedimentation equilibrium are shown in Fig. 4. In order to avoid errors in evaluating x and dc/dx at the bottom of the cell due to the pile-up of sedimented material, the calculation was made from the photograph taken at an early stage of the operation. Fig. 5 shows the enlarged tracing used for the calculation of the molecular weight. The tracing represents the 54 minutes exposure of Fig. 4. The molecular weight was calculated

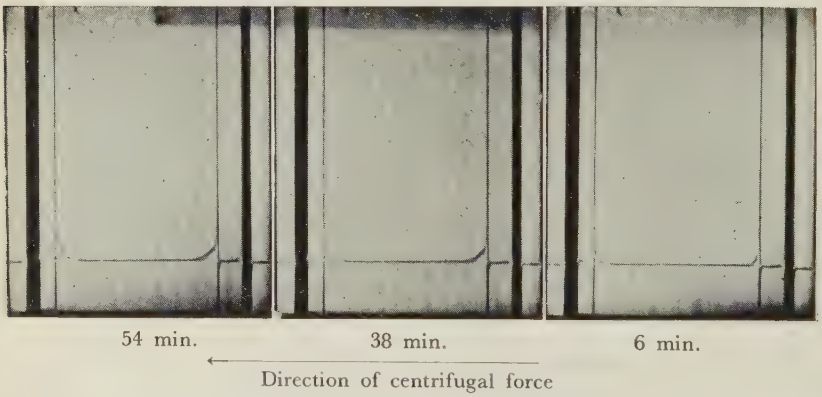

Fig. 4. Ultracentrifugal patterns of saliva-parotin-A during the approach to sedimentation equilibrium. Protein concentration, 1 g. per 100 ml.; rotor speed, 12,590 r.p.m.; angle of phase plate, 70°.

by the following way. The vertical distances, z_n, between the curves and the base line were evaluated at millimeter intervals starting with the meniscus which is expressed as $n=0$. These distances are proportional to dc/dx with the proportionality constant including the optical constants of the ultracentrifuge, the concentration and refractive index increment of the solute and the magnification factor of the enlarger. Since the molecular weight is expressed as the quotient of the terms including *dc/dx*, their evaluation is not necessary.

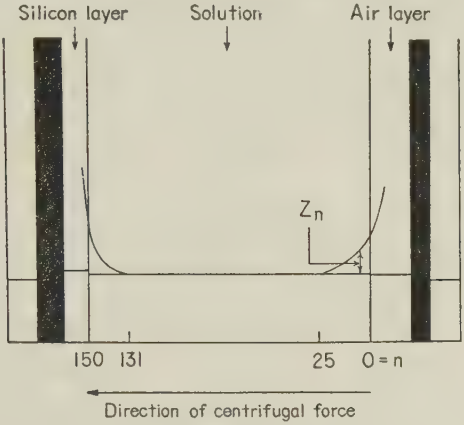

FIG. 5. Enlarged tracing used for the calculation of the molecular weight. This represents the 54 minutes exposure of Fig. 4.

From the values of x and z_n, the terms unknown in the above-mentioned equations are obtained to be

$$\frac{1}{x_b}\left(\frac{dc}{dx}\right)_b=0.371, \qquad \frac{1}{x_m}\left(\frac{dc}{dx}\right)_m=0.298$$

$$\frac{1}{x_b{}^2}\int_X^{x_b} x^2\frac{dc}{dx}dx=\frac{1}{x_b{}^2}\,\frac{dx}{F}\sum_{n_X=131}^{n_b=150} x_n{}^2 z_n=0.0994$$

$$\frac{1}{x_m{}^2}\int_{x_m}^{X} x^2\frac{dc}{dx}dx=\frac{1}{x_m{}^2}\,\frac{dx}{F}\sum_{n_0=0}^{n_X=25} x_n{}^2 z_n=0.1125$$

where F (=13.06) is the magnification factor relating distances in the cell to distances on the enlarged tracing. The molecular weight is thus calculated to be 15,700.

DISCUSSION

As is pointed out by Ginsburg, Appel and Schachman (*5*), there is as yet no adequate theoretical expression for the shape of the curve of (*dc/dx*) *versus* x at the both ends of the cell, and therefore the drawing of the curve is somewhat arbitrary. This may result in the difficulty in the accurate evaluation of z_n on the enlarged tracing. Moreover, there is also some difficulty in the determination of the proper positions of the air-solution and solution-silicone oil interfaces, which may result in the inaccuracy in the estimation of $(dc/dx)_m$ and $(dc/dx)_b$. Although some experimental error may be included in the sedimentation and diffusion coefficients, its effect on the molecular weight would be less than that due to the above-mentioned difficulties. M_{sD}, therefore, may generally be more reliable than M_e. In the present work, however, the value of 15,700 for M_e agrees well with that of 15,900 for M_{sD}. The slight difference between these values would be unimportant taking into account the limitations of accuracy of the methods employed.

The amino acid composition of saliva-parotin-A determined by microbiological method (*4*) indicates that its minimal molecular weight is 15,870 as shown in Table I. This value is consistent with those of M_{sD} and M_e.

TABLE I

Calculation of the Minimal Molecular Weight of Saliva-Parotin-A

Amino acid*	G. amino acid per 100 g. protein	G. amino acid residue per 100 g. protein	No. of residues to nearest integer for M. W. 15,900	G. amino acid residue per g. mole at M. W. 15,900
Alanine	1.00	0.80	2	142.2
Arginine	7.14	6.40	7	1093.3
Aspartic acid	12.70	10.98	16	1841.4
Glutamic acid	22.25	19.25	26	3356.9
Glycine	11.13	8.46	25	1426.3
Histidine	2.47	2.18	3	411.4
Isoleucine	1.90	1.64	2	226.3
Leucine	3.48	3.00	4	452.6
Lysine	4.88	4.28	6	769.6
Phenylalanine	2.08	1.85	2	294.3
Proline	13.72	11.57	20	1942.2
Serine	7.98	6.61	13	1132.0
Threonine	14.83	12.59	21	2123.1
Tyrosine	1.42	1.28	1	163.2
Valine	3.15	2.67	5	495.7
Tatal	110.1	93.8	153	15,870

* Amide groups, the number of which has not yet been precisely determined, are omitted from the calculation. If all the free carboxyl groups of aspartic acid and glutamic acid were substituted by acid amide groups, the minimal molecular weight is calculated to be 15,830.

The molecular weight and frictional ratio of parotin which was isolated from bovine parotid glands (*7*) are 132,000 and 2.21, respectively (*8*). Despite of the similarity of biological activities (*3*), it may be obvious from these data that saliva-parotin-A is quite different from parotin so far as the molecular size is concerned.

The value of 0.688 for the partial specific volume of saliva-parotin-A is considerably low compared with the values of other proteins which are mostly found between 0.73 and 0.75. This may be attributed to the high contents of aspartic acid, glutamic acid, glycine and threonine in the molecule of saliva-parotin-A, because of the low values of the partial specific volumes of these amino acids (aspartic acid, 0.59; glutamic acid, 0.66; glycine, 0.64 and threonine, 0.70). This low value of the partial specific volume would indicate the compactness of the molecule of saliva-parotin-A.

SUMMARY

The molecular weight of saliva-parotin-A was computed to be 15,900, based on a sedimentation coefficient of 1.51×10^{-13} and a diffusion coefficient of 7.41×10^{-7}. The molecular weight determined during the approach to sedimentation equilibrium was 15,700. These values were shown to be consistent with the minimal molecular weight calculated from the amino acid composition.

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INHIBITORY EFFECT OF MOLECULAR NITROGEN ON THE HYDROGEN EVOLUTION BY A BACILLUS CLOSELY RESEMBLING BACILLUS POLYMYXA

By SEIICHI HINO*

(*From the Biological Institute, Faculty of Science,*
Nagoya University, Nagoya)

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Various relationships have been shown to exist between nitrogen fixation and the metabolism of molecular hydrogen. These include: (a) hydrogenase is present in every nitrogen fixing organism which has been tested for the enzyme (*1***, *2–5*), (b) the hydrogenase content of *Azotobacter* (*6*) and *Rhodospirillum* (*7*) is much higher in the cells grown on molecular nitrogen than in the cells grown on ammonium salts or other nitrogen sources and (c) nitrogen fixation by various organisms is competitively inhibited by molecular hydrogen (*1***, *3*, *8*). The underlying mechanism of these relationships still remains obscure.

In 1949, Gest and Kamen (*9*) demonstrated that molecular nitrogen inhibits the photoevolution of hydrogen by *Rhodospirillum rubrum* and this finding lead to the discovery that this organism fixes molecular nitrogen (*10*). Simultaneous experiments showed, however, that ammonia in a low concentration also inhibits the photoevolution, so that it is not clear whether the inhibition is caused by molecular nitrogen *per se* or after its conversion to ammonia which is presumed to be the most likely intermediate in biological nitrogen fixation. Subsequent study by Mortenson and Wilson (*11*) could not detect any effect of molecular nitrogen on the hydrogen evolution from pyruvate by *Clostridium pasteurianum.*

During the studies on the nitrogen fixation by a facultative aerobic bacterium, it was found that the nitrogen fixation took place only under anaerobic condition (*4*). It appeared that nitrogen might have some specific effect on the anaerobic process of the organism. Attempts have been made to examine a possible effect of molecular nitrogen on the evolution of hydrogen, since molecular hydrogen is one of the main products of the anaerobic breakdown of carbohydrates (*4*) and also since molecular hydrogen is a competitive inhibitor of the anaerobic nitrogen fixation by this bacterium (*8*).

Results will be presented in this paper showing that hydrogen evolution from glucose by our bacterium is inhibited by molecular nitrogen and not by ammonium salts or amino acids. Additional information concerning the nature of the hydrogen producing system and its interaction with various nitrogen compounds will also be described.

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* Present address: Laboratory of Biological Science, Department of General Education, Nagoya City University, Nagoya.

** A review containing the references before 1956.

METHODS

Organism—The organism used in this study was originally isolated from soil and described as a strain of *Clostridium* (*12*). Subsequent taxonomical studies showed that it is a facultative aerobic bacillus, which closely resembles *Bacillus polymyxa* and fixes molecular nitrogen only under anaerobic condition (*4*). Effects of various inhibitors on the nitrogen fixation by this bacterium have been reported (*8*, *12*).

Cultivation of the Organism—Under the atmosphere of nitrogen, the organism was grown in a modified Winogradsky's medium with constant shaking at 30°. The medium contained sucrose, 20 g.; $MgSO_4 \cdot 7H_2O$, 500 mg.; $CaCl_2 \cdot 2H_2O$, 100 mg.; $FeSO_4 \cdot 7H_2O$, 15 mg.; NaCl, 10 mg.; $Na_2MoO_4 \cdot 2H_2O$, 5 mg.; biotin, 10 μg.; para-aminobenzoic acid, 10 μg.; distilled water, 500 ml. and K_2HPO_4-KH_2PO_4 buffer (0.1 M, pH 7.7) 500 ml. The buffer solution was sterilized separately and added aseptically just before inoculation of the medium. Cultures were maintained by inoculating into the fresh medium every 24 hours.

Resting Cell Suspension—About 1 ml. of the culture grown for 24 hours was inoculated into 20 ml. of the culture solution in a 200 ml. shake flask. After about 16 hours when the bacteria were in a late logarithmic growth phase, cells from two to three flasks were harvested, washed once with cold 0.01 M phosphate buffer (pH 6.6) and resuspended in 1.5 to 2.0 ml. of the same buffer. All procedures for harvesting and washing the cells were carried out in a cold room.

Gases—Commercial pure nitrogen or argon, both containing less than 0.01 per cent oxygen was used without further purification. The procedures for preparation and purification of hydrogen gas were described in a previous paper (*8*).

Assay of Hydrogen Evolution—Hydrogen evolution was determined at 30° by measuring the changes of manometric readings after CO_2 was absorbed by 0.2 ml. of 20 per cent KOH solution placed with a filter roll in the center well of Warburg vessel. Small pieces of solid pyrogallol were added in the well previous to the addition of the alkali, because the hydrogen evolution by the bacterium is very sensitive to oxygen. Since CO may be evolved from alkaline pyrogallol, some of the results were checked with sodium hydrosulfite-anthraquinone beta-sulfonate. The Warburg vessels were connected to the vacuum line, evacuated to 0.1 atm., filled with oxygen free gases and this procedure was repeated three more times. Cell suspensions (0.2 or 0.3 ml.) placed in the side arm were mixed into the main chamber after taking blank readings to make sure that no oxygen was taken up by cells or pyrogallol. Final fluid in the main vessel contained 0.05 M, pH 6.6 K_2HPO_4-KH_2PO_4 buffer and 0.02 per cent $MgSO_4 \cdot 7H_2O$ in total volume of 2.0 or 3.0 ml. During the bacterial decomposition of glucose, pH of the experimental solution lowered gradually, sometimes to 5.8, but the general feature of the results was unchanged when the initial pH was raised to 7.6 or lowered to 6.0. When the addition of nitrogenous compounds was necessary, they were added in a concentration of 0.015 M on the basis of nitrogen content.

Absorption of hydrogen gas was carried out by palladium black together with 0.5 per cent sodium picrate solution, which is generally used in combination with colloidal palladium (*13*).

RESULTS

Hydrogen Evolution from Various Substrates—It was reported (*4*) that fermentation tests in a growth condition of the present bacterium revealed gas production from a number of carbohydrates. Gasmetric analysis (*8*) showed the presence of molecular hydrogen in the gas phase of culture flasks after bacterial growth under anaerobic condition on sucrose. Present manometric experiment using washed cell suspensions confirmed hydrogen production from

TABLE I

Survey of Various Substances for Hydrogen Evolution

H_2 produced from	H_2 not produced from	
Sucrose	Lactose[1]	Glycerol[1]
Fructose	Arabinose[1]	Lactate
Glucose	Xylose	Acetate
Pyruvate	Gluconate	Glutamate
Formate	Fructose-diphosphate	α-Ketoglutarate
	α-Glycerophosphate	Hypoxanthine

1) These are taken from the previous report (*4*).
The final concentration of each substrate was 0.01 *M*. The vessel containing glucose or without added substrate was used as positive or negative control for the survey.

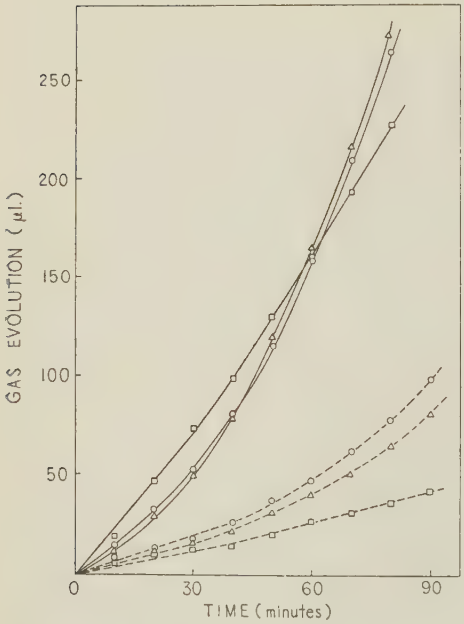

FIG. 1. Effect of aeration on the hydrogen evolution from glucose, pyruvate and formate. Standard system: Side arm; cell suspension. Center well; 20% KOH plus pyrogallol. Main compartment; 0.02% $MgSO_4 \cdot 7H_2O$, 0.05 *M* K_2HPO_4-KH_2PO_4 buffer (pH, 6.6) and substrate. In this experiment, cell suspension was divided into two aliquots, one immediately brought under anaerobic condition, while the other shaken in air for 10 minutes at 30°. Twice amount of cells was used for formate. Concentration of substrate: glucose, 0.02 *M*; pyruvate, 0.04 *M*; formate, 0.02 *M*. Total volume: 2.0 ml. Temperature: 30°. Gas phase: argon.

aerated cells:
- -○- - glucose - -△- - pyruvate
- -□- - formate

control:
—○— glucose —△— pyruvate
—□— formate

sucrose, glucose and fructose. Only pyruvate and formate showed positive hydrogen evolution among various substances that were tested (Table I). The

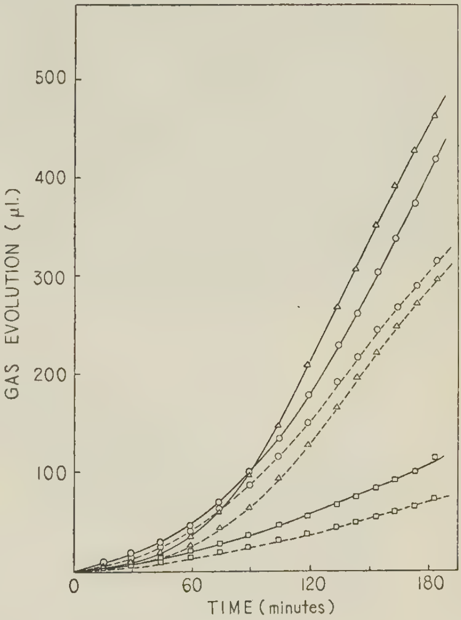

FIG. 2. Hydrogen evolution from glucose, pyruvate and formate under argon and hydrogen. Standard system: see the description in Fig. 1. Concentration of substrate: glucose, 0.01 *M*; pyruvate, 0.02 *M*; formate, 0.01 *M*. Total volume: 3.0 ml. pH: 6.6. 30°.

—○— glucose under Ar.
—△— pyruvate under Ar.
—□— formate under Ar.
- -○- - glucose under H_2.
- -△- - pyruvate under H_2.
- -□- - formate under H_2.

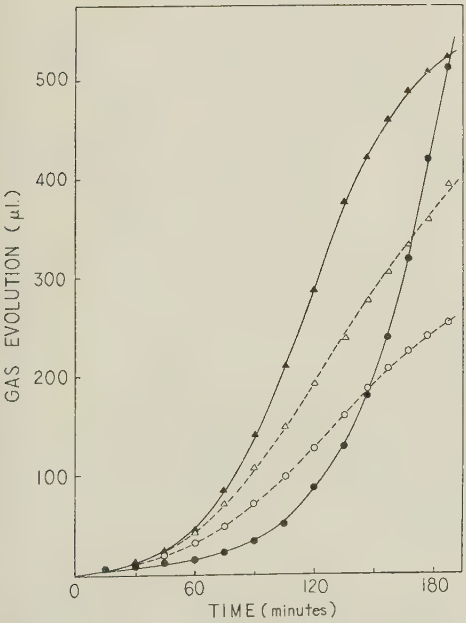

FIG. 3. Hydrogen evolution from glucose and pyruvate under nitrogen and hydrogen. Standard system: see the description in Fig. 1. Concentration of substrate: glucose, 0.01 *M*; pyruvate, 0.02 *M*. Total volume: 3.0 ml. pH: 6.6. 30°.

—●— glucose under N_2.
—▲— pyruvate under N_2.
- -○- - glucose under H_2.
- -△- - pyruvate under H_2.

rate of hydrogen evolution from glucose was very little influenced by the addition of 0.005 M each of lactate, succinate, acetate, hypoxanthine, fructose-diphosphate and 0.002 M of acetaldehyde.

The evolution of hydrogen from glucose or pyruvate is not linear with time but increases gradually during the experiment (Fig. 1). The hydrogen evolution from formate has somewhat different feature; generally the rate of the evolution is distinctly lower than that from the other two substrates and the rate remains constant throughout the course of the experiment. The amount of hydrogen produced from one mole of glucose, pyruvate and formate was about 1.5, 0.5 and 1.0 mole respectively.

Stability of Hydrogen Producing System—When the cells were separated from a supernatant culture solution, washed and recombined with the original supernatant, the rate of hydrogen evolution was considerably lower than the rate from intact culture solution containing the same amount of cell. In the

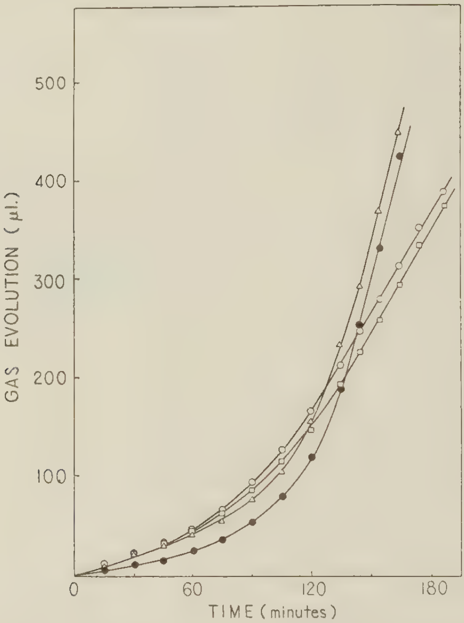

FIG. 4. Effect of glutamate, ammonia and molecular nitrogen on the hydrogen evolution from glucose. Using Warburg vessel with two side arms, cell suspension was placed in one side arm, glucose solution in the other. Glutamate or NH_4Cl (final, 0.015 M) was placed in the main compartment and had been contact with cells for one hour previous to the addition of glucose at zero time. Glucose: 0.02 M. Total volume: 3.0 ml. pH: 6.6. 30°.

—△— NH_4Cl and Ar.
—□— Na-glutamate and Ar.
—●— N_2.
—○— Ar (control).

experiment illustrated in Fig. 1, a washed cell suspension was divided into two aliquots, one immediately brought under anaerobic condition while the other shaken in air at 30° for ten minutes and then brought into anaerobic condition. As the result shows, the cells exposed in air remarkably decreased the ability to produce hydrogen from glucose, pyruvate and formate. Washed cell suspensions, kept overnight in a refrigerator under 100 per cent nitrogen or hydrogen were completely inactive. Various reducing substances, such as ascorbate, glutathione, cysteine or hydrogen sulfide were all without effect for preventing the inactivation or reactivation of cells once inactivated.

Effect of Molecular Hydrogen on the Hydrogen Evolution from Glucose, Pyruvate and Formate—Since molecular hydrogen is a competitive inhibitor of the anaerobic nitrogen fixation by this organism, its effect on the hydrogen evolution from glucose, pyruvate and formate was examined. The result is shown in Fig. 2 and Fig. 3. Hydrogen inhibited the rate of gas evolution from pyruvate and formate by 30 to 40 per cent when either argon or nitrogen was used as a control. Similar inhibition was obtained with glucose using

TABLE II

Effect of Various Nitrogenous Compounds on the Hydrogen Evolution from Glucose

Expt. no.	Gas phase	N-source	H_2 produced from glucose during		
			0–30 min.	90–120 min.	120–150 min.
I	Ar	Glutamate	20.1 μl.	62.8 μl.	81.5 μl.
		NH_4Cl	21.8	72.8	173.5
		None	22.4	73.3	95.8
	N_2	None	14.9	65.5	175.5
		α-Ketoglutarate	14.3	60.4	155.5
II	Ar	Aspartate	33.1	109.3	
		Glycine	35.6	98.1	
		None	32.8	80.7	
	N_2	None	23.0	158.9	
III	Ar	Histidine	24.5	83.6	
		Hypoxanthine	25.5	114.2	
		NH_4Cl	31.5	166.5	
		None	28.4	109.5	
IV	Ar	Asparagine	31.0	58.3	
		NH_4Cl	37.0	75.0	
		None	32.2	63.4	
	N_2	None	22.8	50.1	
V	Ar	NH_4Cl	31.1	56.5	
		$(NH_4)_2SO_4$	28·8	67.7	
		None	29.7	55.0	
	N_2	None	20.5	43.8	

Hydrogen evolution was measured by Warburg vessels at 30° after CO_2 was absorbed by 20% KOH (with filter paper). Suspensions of about 16 hour cells were placed in the side arm. Reaction mixture contained final concentration of 0.02 M glucose, 0.02% $MgSO_4 \cdot 7H_2O$, 0.05 M K_2HPO_4-KH_2PO_4 buffer (pH 6.6) and 0.015 M (N-equivalent) nitrogenous compounds in total volume of 2.0 or 3.0 ml. The data given under Expt. I are taken from the same experiment drawn in Fig. 4.

argon as a control. However, a situation was different when the hydrogen evolution from glucose was compared under nitrogen and hydrogen: apparently there was initial stimulation followed by increasing inhibition by molecular hydrogen. It was inferred from the results that hydrogen itself inhibited the hydrogen evolution from these three substrates to a similar extent while nitrogen had some specific effect on the gas evolution from glucose.

Stimulation of the Hydrogen Evolution by Molecular Nitrogen and Ammonia—When the effect of molecular nitrogen on the hydrogen evolution from glucose was examined using argon as a control, apparent inhibition by nitrogen appeared in the earlier part followed by a remarkable stimulation in the later part of the gas evolution. The effect of other nitrogenous compounds was then tested and the result is shown in Fig. 4 and Table II. In the experiment illustrated in Fig. 4, ammonia or glutamate was incubated with cells for one hour previous to the addition of glucose. The stimulating effect in the later part of hydrogen evolution also appeared with ammonia but no initial inhibition was observed. Glutamate, aspartate, glycine, histidine, hypoxanthine and asparagine showed neither significant inhibition nor stimulation.

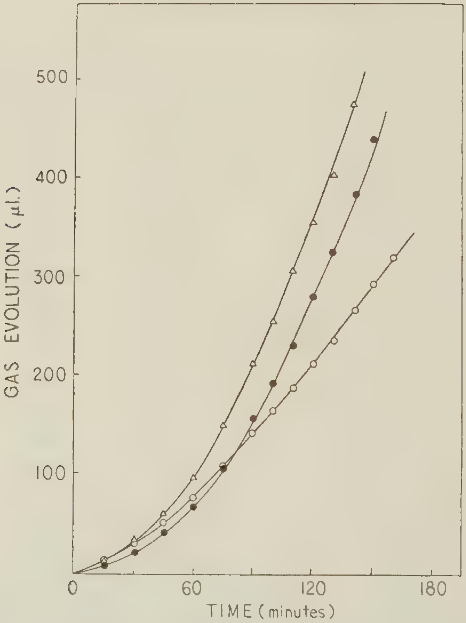

FIG. 5. Effect of ammonia and molecular nitrogen on the hydrogen evolution from pyruvate. Standard system: see the description in Fig. 1. Pyruvate: 0.04 *M*. Total volume: 2.0 ml. pH: 6.6. 30°.

—●— N_2. —○— Ar.

—△— NH_4Cl (0.015 *M*) and Ar.

When pyruvate was used as a substrate, there was a slight initial inhibition by molecular nitrogen, but the inhibition was never so distinct as in the case of glucose breakdown. When appropriate amount of cells (about 100 to 200 μg. nitrogen per manometer flask) taken from actively growing cultures were used, it was possible to observe gradual increase of the rate of hydrogen evolution by the action of molecular nitrogen* and here again similar increase of the rate could be observed with ammonia (Fig. 5). The stimulating effect by ammonia and nitrogen may not be caused by cell multiplication, because the bacteria showed little or no growth after 24 hours under anaerobic incubation with pyruvate as sole source of carbon.

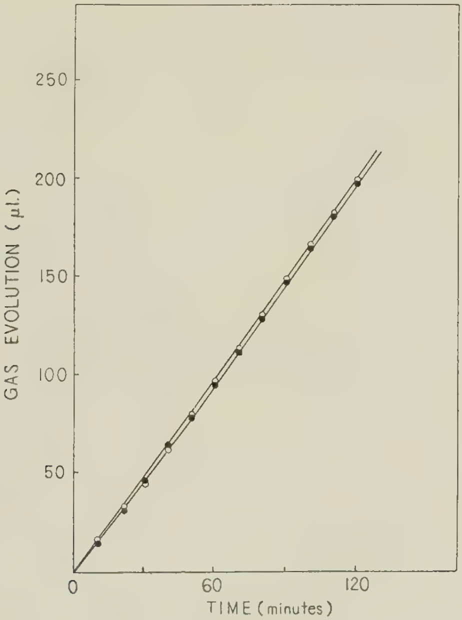

Fig. 6. Hydrogen evolution from formate under argon and molecular nitrogen. Standard system: see the description in Fig. 1. Formate: 0.01 *M*. Total volume: 2.0 ml. pH: 6.6. 30°.

—●— N_2. —○— Ar.

The effect of molecular nitrogen on the hydrogen evolution from formate was investigated and the result (Fig. 6) shows that nitrogen had no inhibitory nor accelerating effect.

Inhibition by Molecular Nitrogen of the Hydrogen Evolution from Glucose—The experimental results so far described in this paper reveal that distinct inhibition of hydrogen evolution appeared only when glucose was used as a substrate and molecular nitrogen, among the various nitrogenous compounds tested, as an inhibitor. The system responsible for this nitrogen inhibition is susceptible to various treatments and special care was taken in order to get reproducible results. It was necessary to use a rather heavy suspension of the cells (about 200 to 300 μg. nitrogen per manometer flask) that were harvested from the culture actively fixing nitrogen and used for the experiment immediately after washing. It was also necessary to keep the glucose concentration between $10^{-2.5}$ and $10^{-1.5}$ *M*. Nitrogen inhibition was not distinct with the cells taken from cultures in a stationary growth phase or the cells grown on ammonia. A typical result obtained after the establishment of the experimental condition is shown in Fig. 7.

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* In the experiment shown in Fig. 3, pyruvate had been consumed before appreciable acceleration by molecular nitrogen occurred.

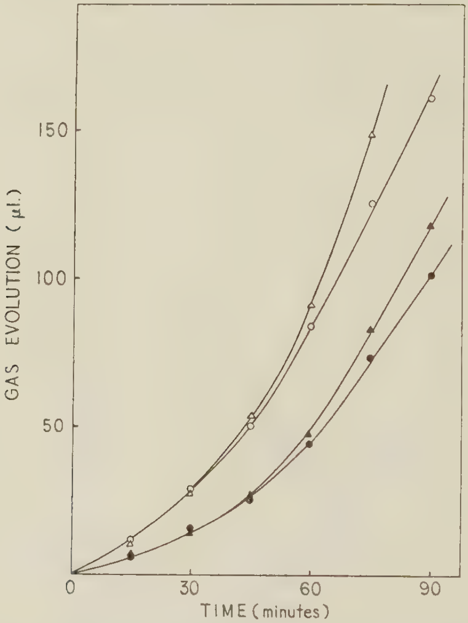

FIG. 7. Inhibition by molecular nitrogen of the hydrogen evolution from glucose. Standard system: see the description in Fig. 1. Cells grown for 15 hours and containing about 200 μg. nitrogen were used to each vessel. Glucose: 0.02 *M*. NH_4Cl: 0.015 *M*. Total volume: 3.0 ml. pH: 6.6. 30°.

—▲— NH_4Cl and N_2. —●— N_2.
—△— NH_4Cl and Ar. —○— Ar.

Inhibition of the photoevolution of hydrogen by *Rhodospirillum rubrum* is brought about not only by molecular nitrogen but also by ammonia (*9*). Since in our experiment, ammonia had an accelerating effect on the hydrogen evolution from glucose, it was suspected that this acceleration might mask the initial inhibition. That such is not the case is demonstrated in Fig. 7 which shows that inhibition by molecular nitrogen could not be reversed by the addition of ammonia. The use of different concentration ($10^{-3.5}$ to $10^{-2.0}$ *M*) of ammonia or the previous contact of cells with it also failed to show any inhibition.

In the experiments so far reported, manometric changes after absorption of carbon dioxide by alkali were regarded as caused solely by molecular hydrogen. As the organism used is a nitrogen fixer, the possibility was examined whether the apparent decrease of gas evolution from glucose under nitrogen might be due to the absorption of nitrogen rather than the inhibition of hydrogen evolution. Manometric changes were measured using six flasks containing the same reaction mixture and filled with either argon or nitrogen. Palladium black and picrate solution were added in the second side arm of

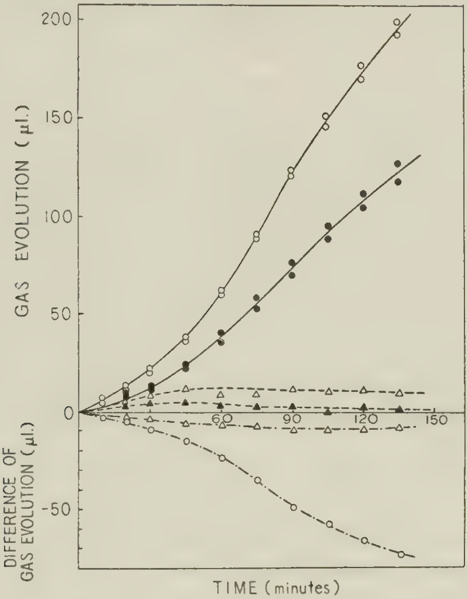

FIG. 8. Absorption of hydrogen produced from glucose under argon and nitrogen by palladium. Cells grown for 16 hours and containing about 200 μg. nitrogen were used for each vessel. CO_2 was absorbed by 20% KOH—see the description of Fig. 1 for standard system. Glucose: 0.02 *M*. Total volume: 2.0 ml. pH: 6.6. 30°.

A: ——○—— Gas evolution under Ar (in duplicate).

B: ——●—— Gas evolution under N_2 (in duplicate).

B – A: —·–○–·— Difference of gas evolution under N_2 and Ar.

C: – – –△– – – Gas evolution under Ar when Pd was present.

D: – – –▲– – – Gas evolution under N_2 when Pd was present.

D – C: —·–△–·— Difference of gas evolution when Pd was present.

In C and D, 500 mg. of palladium black and 0.2 ml. of 0.5 per cent Na-picrate were present in the second side arm of Warburg vessel.

each one of the flasks containing the different kind of gases. The result (Fig. 8) indicates that more than 95 per cent of the alkaline insoluble gas, produced from glucose under both argon and nitrogen, was absorbed by palladium. It is also shown that the difference of manometric readings between the flask containing nitrogen and argon was far less when palladium was present.

This result indicates that the hydrogen evolution from glucose was actually inhibited by nitrogen and also that very little nitrogen fixation, if any, occurred during the experiment. Amount of nitrogen absorbed by cells did not exceed 10 microliters or it was less than 0.5 μmoles in total volume of 2.0 ml. of the reaction mixture at the end of the experiment.

DISCUSSION

Two main routes are known to exist that are responsible for the production of molecular hydrogen from carbohydrates by various microorganisms (*14*). One is the formic hydrogenlyase system generally found in coli-aerogenes bacteria and the other is the direct phosphoroclastic formation of hydrogen from pyruvate generally found in *Clostridium*. Since with our bacterial strain, not only hydrogen was found to be produced from formate but also the accumulation of formate could be detected in a culture fluid actively fermenting carbohydrates*, it is likely that a formic hydrogenlyase is responsible for the formation of hydrogen from carbohydrates, although the simultaneous presence of other system such as the one found in *Clostridium* can not be excluded.

One of the characteristics of the hydrogen-producing system in the present bacterium is its extreme sensitivity to oxygen. Although special care was taken to avoid the inactivation during the preparation of resting cell suspensions, considerable activity had to be lost during the treatment. Crewther (*15*), using a strain of *Bacillus polymyxa*, reported that the formic hydrogenlyase of this bacterium is more sensitive to oxygen than that of *Escherichia coli*.

It was observed by Pinsky and Stokes (*16*) that aerobically grown cells of *Escherichia coli* did not show any hydrogen production during the anaerobic incubation with formate alone, but simultaneous addition of both glucose and suitable nitrogen sources caused gradual appearance of the formic hydrogenlyase activity. In our bacterium, the rate of hydrogen formation from formate remains constant on a low level while that from glucose or pyruvate shows gradual increase which is more pronounced in the presence of ammonia or molecular nitrogen. This effect may be explained that, analogous to the result obtained by Pinsky and Stokes (*16*), glucose (or pyruvate) and ammonia (or nitrogen) are effective for the reformation of formic hydrogenlyase activity that is partially lost during the preparation of resting cell suspensions.

The evolution of molecular hydrogen from glucose by the present organism is inhibited by molecular nitrogen but not by ammonia or other nitrogen compounds that were tested. The data presented in Fig. 8 show that only very small amount of molecular nitrogen was consumed when distinct inhibition of hydrogen evolution appeared. These results make it very probable that the inhibition is caused by molecular nitrogen itself rather than by a product of nitrogen fixation.

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* Unpublished experiment by S. Hiai of this laboratory

A close relationship between nitrogen fixation and hydrogenase has been repeatedly suggested by Wilson and his colleagues (for example *1*), on the basis of competitive inhibition by molecular hydrogen of nitrogen fixation and characteristic increase of hydrogenase activity in the cells grown on molecular nitrogen. Our finding that molecular nitrogen does inhibit hydrogen evolution provides another implication between hydrogenase and nitrogen fixation. The simplest assumption that in the present bacterium molecular nitrogen inhibits hydrogenase activity remains to be subjected to future examination, because molecular nitrogen does not inhibit hydrogen evolution from pyruvate or formate in contrast to oxygen and hydrogen which inhibit hydrogen evolution from these two substrates as well as glucose.

Although the underlying mechanism of nitrogen inhibition is not known at present, our result would give a clue to correlate nitrogen fixation with the process of carbohydrate metabolism. It would also be worth mentioning that most of the studies on nitrogen fixation are carried out by the Kjeldahl analysis of total nitrogen after organisms were kept under growth condition. Manometric method without significant cell multiplication may offer an experimental method for the study of biological nitrogen fixation.

SUMMARY

1. The effect of molecular nitrogen and several other nitrogenous compounds on the evolution of molecular hydrogen was studied using cell suspensions of a facultative aerobic bacterium, which closely resembles *Bacillus polymyxa* and fixes nitrogen only under anaerobic condition.

2. The organism produces hydrogen from sucrose, glucose, fructose, pyruvate and formate. No hydrogen is produced from several other substances such as fructose-diphosphate, gluconate, lactate or hypoxanthine.

3. The rate of hydrogen evolution from glucose or pyruvate gradually increases with time, while the rate of evolution from formate remains on a constant low level. Hydrogen or oxygen inhibits the hydrogen evolution from these three substrates to a similar extent.

4. Molecular nitrogen has a dual effect on the hydrogen evolution from glucose; initial inhibition followed by a remarkable stimulation. Under the condition in which the inhibitory effect of molecular nitrogen could clearly be demonstrated, ammonium salts, glutamate and several other amino acids did not show any such inhibition.

5. No initial inhibition by molecular nitrogen could be demonstrated on the hydrogen evolution from pyruvate or formate, although the stimulating effect of nitrogen could be observed with pyruvate. Ammonia also showed

the stimulating effect on the hydrogen evolution from glucose and pyruvate.

The author wishes to express his sincere gratitude to Prof. T. Mori for his continuous encouragement and advices. He is also grateful to Mr. S. Hiai for his valuable discussions. The expences for this work were partly defrayed by the Grant in Aid for the Scientific Research from the Ministry of Education.

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ACETYL COENZYME A DEACYLASE IN LIME FRUIT (CITRUS MEDICA LINN VAR ACIDA)

By S. S. TATE, C. V. RAMAKRISHNAN AND N. T. PATEL

(*From the Department of Biochemistry, Faculty of Science, M. S., University of Baroda, Baroda, India*)

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The accumulation of citric acid in the mature lime fruit (*Citrus medica Linn Var acida*) and its absence in the young fruit has been observed from recent studies in this laboratory (*1*). While the accumulation of citric acid in the former could be explained in terms of a block in the operation of the tricarboxylic acid cycle at the citrate level because of the absence of aconitase activity, and in terms of the increased formation of citrate and its precursors because of changes in the activities of related enzymes, the absence of citric acid in the young fruit despite the presence of enzymes like the condensing enzyme, malic dehydrogenase, pyruvic oxidase, oxalacetic carboxylase, *etc.* needed explanation. This led the authors to suspect the presence, in young fruits, of enzymes which effect the partial hydrolysis of the precursors of citric acid, namely acetyl coenzyme A and oxalacetate. One such enzyme, oxalacetic hydrolase, which hydrolyses oxalacetate, has already been detected in this laboratory (*2*). A similar breakdown of acetyl Coenzyme A by acetyl Coenzyme A deacylase was also suspected and its presence investigated. This communication reports the detection and purification of acetyl Coenzyme A deacylase in young lime fruits and changes in the activity of this enzyme with the development of the fruit.

EXPERIMENTAL

Assay

The enzyme assay was based on the following reaction :—

$$\text{Acetyl phosphate} + \text{Coenzyme A} \underset{\longleftarrow}{\xrightarrow{\text{Phosphotransacetylase}}} \text{Acetyl Coenzyme A} + \text{Phosphate} \quad (1)$$

$$\text{Acetyl Coenzyme A} + H_2O \xrightarrow{\text{Deacylase}} \text{Acetate} + \text{Coenzyme A} \quad (2)$$

The left over acetyl phosphate was determined by the method of Lipmann and Tuttle (*3*).

Assay for protein content of enzyme preparations :—The protein content of the crude enzyme preparation was estimated by the micro-Kjeldahl method, and that of the purified preparation by measuring the ultraviolet absorption at 260 mμ and 280 mμ (*4*). Citrate was determined by the method of Saffran and Denstedt (*5*).

Materials

Transacetylase, in the form of ammonium sulphate fraction, was prepared from the cell free extracts of *E. coli* N.R.C. 428, as described by Ramakrishnan and Martin (*6*), Coenzyme A was prepared from sheep liver according to the method of Beinert *et al.* (*7*), dilithium acetyl phosphate by that of Avison (*8*), calcium phosphate gel as described by Keilin and Hartree (*9*), and alumina gel as described by Willstätter and Kraut (*10*). All other chemicals were commercial preparations.

Collection of Lime Fruits:—The fruits were collected from a lime farm near Baroda during the same season from a single plot of 150 trees of almost the same age. They were divided into three groups of different sizes each consisting of uniform size as determined by their diameters taken equatorially. The diametrical sizes chosen for investigation were 0.5 cm., 1.5 cm., and 4.2 cm. and represent respectively the stages in which the accumulation of citric acid is absent, has just commenced, and is considerable. The number of fruits used for each trial differed with fruit size, being 200, 50, and 10 respectively for young, middle sized, and matured fruits.

Methods

Preparation of the Enzyme from the Fruit Tissue:—The fruits were brought fresh from the farm in an ice box and the sampling was done immediately in a cold room at 0-4°. The fruits were peeled and seeded and a known weight of the cut tissue was ground for 20 minutes with one third its weight of Alumina (Alcoa A-301, Alumina ore Company of America) and two times its volume of 0.02 *M* cold potassium phosphate buffer, pH 7, in a chilled mortar kept at 0–4°. The pH of the homogenate was maintained at 7 during grinding by adding drops of 6 *M* KOH when necessary. The homogenate was centrifuged at 18,400×*g* for 20 minutes in a refrigerated centrifuge, the temperature being maintained between 0-4°. The clear supernatant obtained was used for detecting the activity of acetyl Coenzyme A deacylase.

Preparation of the Fruit Extract for Citrate Estimation:—The fruits were peeled, seeded, and finely sliced. A known weight of the cut tissue was immediately homogenized in a Waring blendor for five minutes with a known amount (2–3 times) of glass distilled water and was made upto a definite volume. For the estimation of citric acid in the tissue homogenate, a known volume of the homogenate was taken and centrifuged and the clear supernatant obtained was used.

Partial Purification of Acetyl Coenzyme A Deacylase:—For the purification of the enzyme, the crude extract obtained from fruits having a diameter of 0.5 cm. was used. To 30 ml. of cell free extract (A) was added 14.7 g. of solid ammonium sulphate, allowed to stand at 0° for 10 minutes and then centrifuged at 18,400×*g* for 20 minutes at 0–4°. The resulting precipitate was dissolved in 0.02 *M* cold potassium phosphate buffer, pH 7.0 to a volume of 22 ml. (Ammonium sulphate fraction I).

To 22 ml. of Ammonium sulphate fraction I was added 7.7 g. of solid ammonium sulphate, the precipitate removed by centrifugation at 2,040×*g* for 10 minutes, and 3.08 g. of solid ammonium sulphate further added to the supernatant. This was again centrifuged at 18,400×*g* for 10 minutes and the precipitate obtained dissolved in 0.02 *M* cold potassium phosphate buffer, pH 7.0, to a volume of 5.0 ml. (Ammonium sulphate fraction II).

To the residue obtained after centrifugation of 2.5 ml. of calcium phosphate gel (8.0 mg. dry weight per ml.) were added 5 ml. of ammonium sulphate fraction II. The mixture was allowed to stand at 0° for 10 minutes with occasional stirring. It was then centrifuged at 2,040×*g* and the supernatant (calcium gel supernatant) tested for enzyme activity.

Five ml. of the calcium gel supernatant were added to the residue obtained after centrifugation of 2.5 ml. of alumina gel (11 mg. dry weight per ml.). This mixture was allowed to stand at 0° for 10 minutes with occasional stirring, then centrifuged at 2,040×*g* for 10 minutes, and the superatant (Alumina gel supernatant) was saved and tested.

Measurement of Enzyme Activity:—The reaction mixture (total volume 1.5 ml.) contained glycine buffer, pH 6.8, 25 μM; NaF, 2 μM; $MgCl_2$, 4 μM; Coenzyme A (120 units per ml.), 0.15 ml; potassium L-cysteine hydrochloride, pH 7.0, 4 μM; lithium acetyl phosphate, 6 μM; ammonium sulphate fraction of *E. coli.* extract (9.0 mg. of protein per ml.), 0.05 ml. and enzyme preparation. A blank with boiled enzyme preparation in place of fresh enzyme was run side by side.

The additions were made in test tubes kept in ice. After addition of the enzyme, the tubes were incubated at 30° for 40 minutes after which the left over acetyl phosphate was estimated. The difference between the experimental and the blank was taken to indicate the breakdown of acetyl phosphate due to deacylase activity.

Deletion studies were made to determine the dependence of acetyl phosphate breakdown on the individual components of the system.

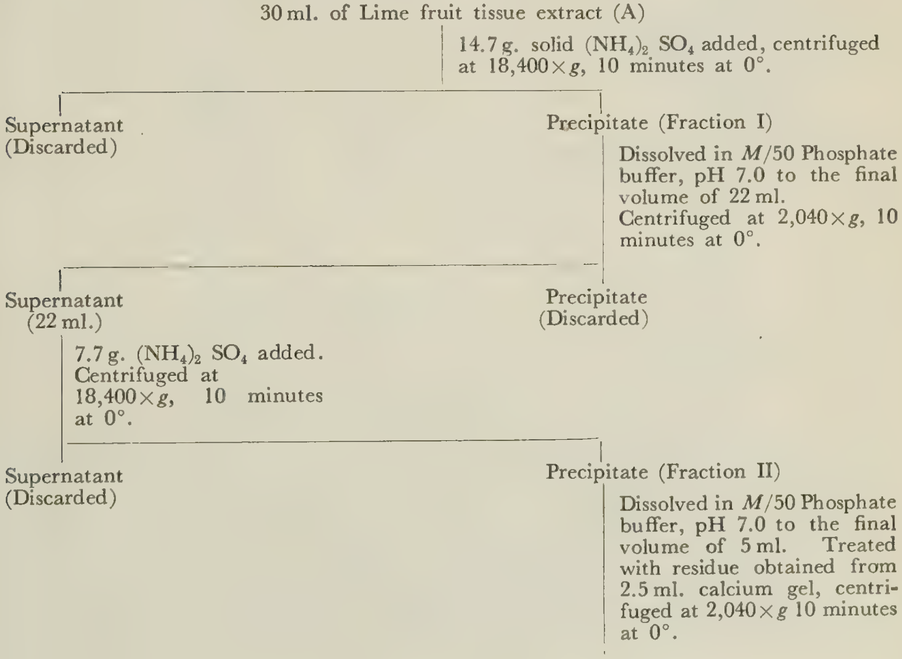
A unit of enzyme activity is defined as the amount of enzyme which under the conditions of the assay catalysed the net disappearance of 1 μM of acetyl phosphate in 40 minutes at 30°. The specific activity is expressed as units per mg. of protein.

RESULTS

The whole procedure of fractionation of the lime fruit tissue extract is shown by the following flow sheet:

Scheme 1

Flow Sheet for Fractionation of Lime Fruit Tissue Extract.


30 ml. of Lime fruit tissue extract (A)
14.7 g. solid $(NH_4)_2 SO_4$ added, centrifuged at 18,400×g, 10 minutes at 0°.
Supernatant (Discarded)
Precipitate (Fraction I)
Dissolved in M/50 Phosphate buffer, pH 7.0 to the final volume of 22 ml. Centrifuged at 2,040×g, 10 minutes at 0°.
Supernatant (22 ml.)
Precipitate (Discarded)
7.7 g. $(NH_4)_2 SO_4$ added. Centrifuged at 18,400×g, 10 minutes at 0°.
Supernatant (Discarded)
Precipitate (Fraction II)
Dissolved in M/50 Phosphate buffer, pH 7.0 to the final volume of 5 ml. Treated with residue obtained from 2.5 ml. calcium gel, centrifuged at 2,040×g 10 minutes at 0°.

Calcium gel Supernatant (5 ml.)

Calcium phosphate gel (Discarded)

Treated with residue obtained from 2.5 ml. Alumina gel
Centrifuged at 2,040×g, 10 minutes at 0°.

Alumina gel Supernatant

Alumina gel (Discarded)

The purification of acetyl CoA deacylase resulted in an increase of about 16 fold in the specific activity of the enzyme in the protein of the alumina gel supernatant (Table I). All the fractions lost their activity on storage.

TABLE I

Purification of Acetyl CoA Deacylase

Fraction	Volume ml.	Total protein mg.	Total activity units.	Recovery of total units %	Specific activity.
Crude extract (A)	30	193.0[1)]	241.0	100.0	1.2
Ammonium sulphate fraction I	22	128.0	238.0	98.6	1.9
Ammonium sulphate fraction II	5	22.3	117.0	48.5	5.3
Calcium phosphate gel supernatant	5	10.4	108.0	44.8	10.4
C^{r} Alumina gel supernatant	5	5.3	105.0	53.6	19.8

1) From 14 gm. fresh weight of fruit tissues.

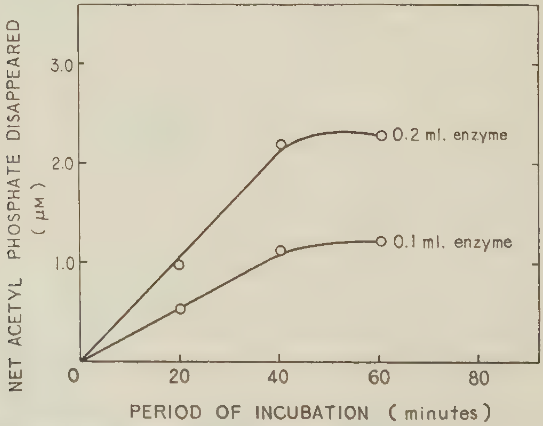


FIG. 1. Effect of time and enzyme concentration on acetyl Coenzyme A deacylase. [$(NH_4)_2SO_4$ Fraction I used.]

The crude preparation lost about half its activity in four days and all its activity in ten days, whereas the purified preparation lost all its activity in 24 hours.

Figures 1 to 9 show the variations in the activity of the enzyme with enzyme concentration and period of incubation, pH, temperature and the concentrations of acetyl phosphate, coenzyme A, *E. coli.* preparation, buffer, magnesium chloride and cysteine. Table 2 shows the dependence of acetyl phosphate breakdown on the various components of the system. It can be seen from the table that the reaction is Coenzyme A dependent. Further, neither *E. coli.* preparation nor deacylase can effect an appreciable breakdown of acetyl phosphate singly, whereas they do so in combination. The optimum

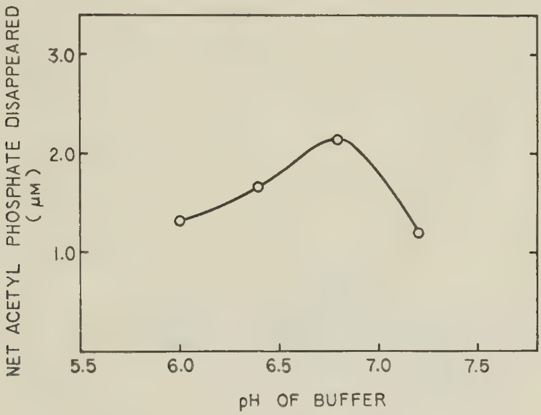


FIG. 2. Effect of pH on acetyl Coenzyme A deacylase.
[0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

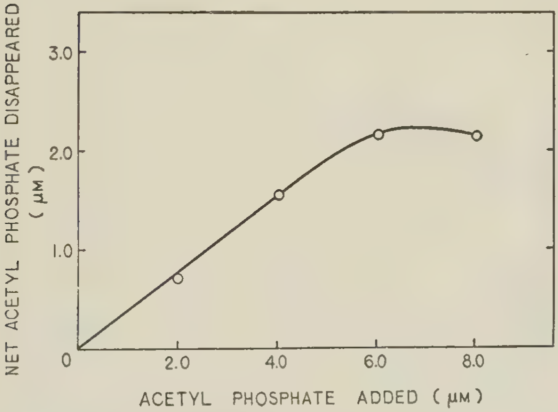


FIG. 3. Effect of substrate concentration on acetyl Coenzyme A deacylase.
[0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

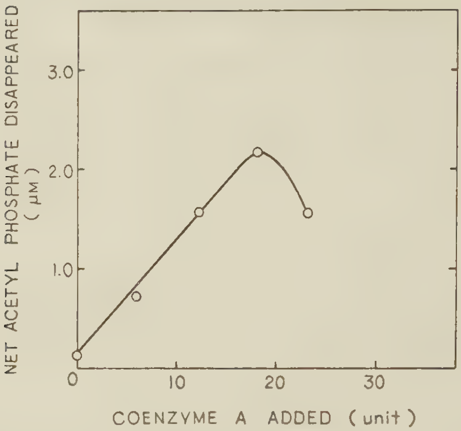


FIG. 4. Effect of coenzyme A concentration on acetyl Coenzyme A deacylase.

[0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

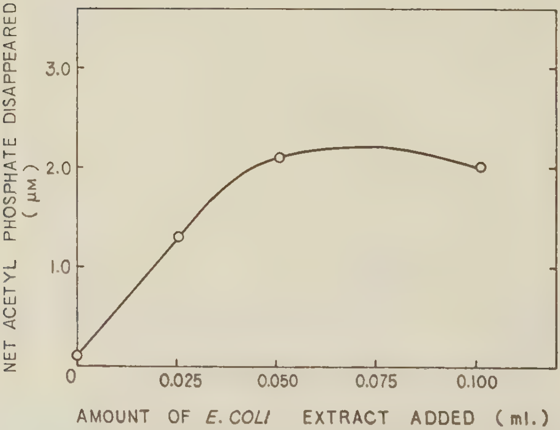


FIG. 5. Effect of concentration of trans-acetylase preparation on acetyl Coenzyme A deacylase.

[0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

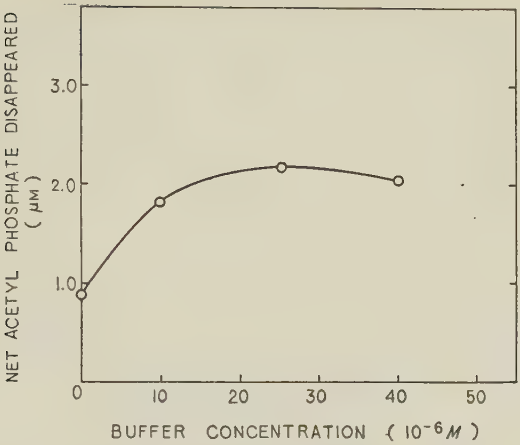


FIG. 6. Effect of concentration of buffer on acetyl Coenzyme A deacylase.

[0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

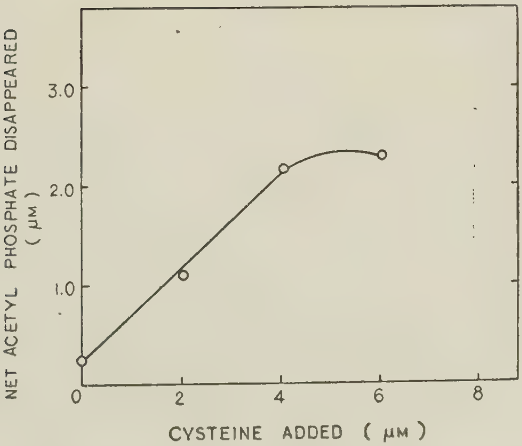


FIG. 7. Effect of cysteine on acetyl Coenzyme A deacylase. [$(NH_4)_2SO_4$ Fraction I used.]

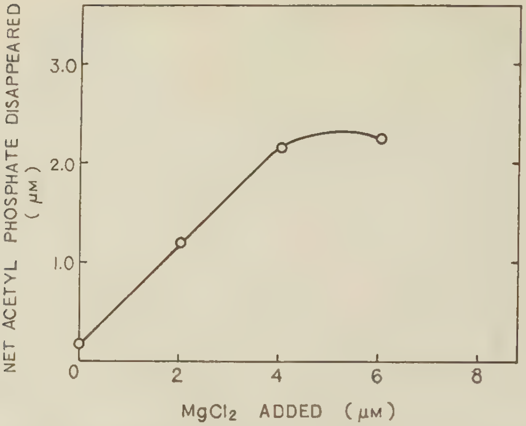


FIG. 8. Effect of $MgCl_2$ on acetyl Coenzyme A deacylase. [$(NH_4)_2SO_4$ Fraction I added.]

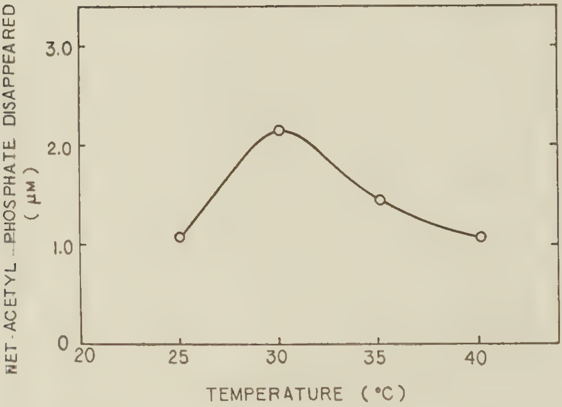


FIG. 9. Effect of temperature on acetyl Coenzyme A deacylase. [0.2 ml. of $(NH_4)_2SO_4$ Fraction I used.]

pH for acetyl Coenzyme A deacylase, shown in figure 2 is 6.8.

Table 3 gives the changes in acetyl Coenzyme A deacylase activity of lime fruit tissue during development on a per fruit basis. It can be seen from the same table that the activity of the enzyme diminishes to zero during the peak period of accumulation of citric acid.

TABLE II

Acetyl Phosphate Breakdown Catalysed by Phosphotransacetylase and Acetyl CoA Deacylase[1)]

Components	Net acetyl phosphate disappearance[2)] (μ Moles)
Complete system	2.16
Deacylase heated at 70° for 5 minutes	0.00
No E. coli extract	0.12
No Coenzyme A	0.15
No cysteine	0.24
No magnesium chloride	0.18

1) 0.2 ml of Ammonium sulphate fraction I was used as a source of enzyme
2) Values were corrected for spontaneous breakdown of acetyl phosphate.

TABLE III

Change in Acetyl Coenzyme A Deacylase Content of Lime Fruit Tissues during its Development

(on a per fruit basis)

Diameter of the fruit (cm.)	Citric acid (g)	Acetyl Coenzyme A deacylase (enzyme units)
0.5	0.00000	12.5
1.5	0.010	45.6
4.2	1.34400	0.0

DISCUSSION

The hypothesis that the non-accumulation of citric acid in lime fruit during the initial stages of development may be partly due to the enzymic breakdown of acetyl Coenzyme A formed derives support from the presence of acetyl Coenzyme A deacylase during non-accumulation of citric acid, and its absence during accumulation.

It thus appears that, in addition to the factors previously reported, namely, the presence of oxalacetic hydrolase and the low activity of condensing enzyme (*2*), the presence of acetyl Coenzyme A deacylase in young fruits contributes to the non-accumulation of citrate in the same by effecting a partial breakdown of the C_2-acid necessary for its formation. The absence of this enzyme during the peak accumulation of citric acid further strengthens this suggestion.

SUMMARY

The enzyme, acetyl Coenzyme A deacylase, has been detected and

purified from cell free extracts of lime fruit tissue during the initial stages of their development. This enzyme is found to require magnesium for maximal activity. The possible role of this enzyme in citrate metabolism has been considered.

The authors' thanks are due to Dr. R. Rajalakshmi for guidance in the presentation of this paper.

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SYNTHESIS AND BIOLOGICAL ACTIVITIES OF MYCOLOYLAMINO ACIDS

By YUICHI YAMAMURA, ATSUSHI TANAKA AND
MASAHIKO KATO*

(*From the Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka*)

(Received for publication, October 23, 1959)

The extensive research on experimental tuberculosis during the past decades has led to the discoveries of several biologically active substances in tubercle bacilli, which seem to contribute much to our understanding of the pathogenesis of tuberculosis. Several chemical substances having such biological activities were shown to have "mycolic acid", a high molecular, branched-chain fatty acid as amain component.

One of these substances is "cord factor", a toxic lipide component of the tubercle bacilli, which was identified as trehalose-6, 6′-dimycolate by the chemical analysis on the purified material and chemical synthesis by Bloch, Noll, Asselineau and Lederer (*1*, *2*). Further, it was reported by Asselineau *et al.* that the synthetic glucosamine or glucose ester of mycolic acid had the cord factor like toxicity to mice while free mycolic acid had no such activity (*3*).

Cord factor showed a particular delayed toxicity for mice upon repeated injections of its small amounts (*4*). Kato *et al.* revealed the fact that the activities of succinic and other diphosphophyridine nucleotide-linked (lactic, α-glycerophosphoric and malic) dehydrogenases in mouse liver remarkably decreased after the intraperitoneal injection of cord factor (*5*).

The adjuvant effect of tubercle bacilli in the induction of experimental encephalomyelitis was attributed to wax D, which is one of the chloroform soluble components of the tubercle bacilli and considered to be a lipopolysaccharide mainly composed of mycolic acid (*6*).

The cavity inducing substance, a proteolipide isolated from tubercle bacilli by Yamamura, was also composed of mycolic acid amounting as much as 70–80 per cent of its lipide component (*7*).

From the above described findings, it should call intereset to recognize the biological and pathological activities of mycolic acid or its derivatives. The present paper describes the methods for synthesizing the conjugates of mycolic acids and several kinds of amino acid and the biological activities of these synthesized mycoloylamino acids on the succinic dehydrogenase of mice liver.

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* Present address; Research Laboratory of the National Sanatorium, Toneyama Hospital, Toyonaka, Osaka.

MATERIALS AND METHODS

Chemicals—Mycolic acid was obtained from human tubercle bacilli, strain $H_{37}Rv$, and BCG according to the method of Asselineau (*8*). Dicyclohexylcarbodiimide (DCD) was a commercial product of Aldrich Chemical Co., Inc.

Mouse—Male animals weighing 15–20 g. of a uniform strain of albino mice-strain-ddN were used for the test of the biological activity of synthesized mycoloylamino acids. Five animals were used in one test group with five control animals.

Determination of the Cord Factor-Like Toxicity of Mycoloylamino acids for Mice—Mycoloylamino acids were dissolved to make 0.1 per cent solution in olive oil and sterilized by heating at 100° for 30 minutes. The occurrence of loss of body weight and death was observed from the successive intraperitoneal injections of 0.1 mg. of each mycoloylamino acids per 0.1 ml. olive oil at four days interval. Control mice received olive oil only.

Assay for the Succinic Dehydrogenase Activity of Mouse Liver—Approximately 24 hours after the intraperitoneal injection of 0.1 mg. of each mycoloylamino acid, the liver of mice was removed and homogenized with 9 volumes of chilled 0.1 *M* phosphate buffer (pH 7.4) using a glass homogenizer of Potter-Elvehjem type. The succinic dehydrogenase activity of the liver homogenate was determined colorimetrically by Kun and Aboad's method (*9*) using triphenyltetrazolium chloride as hydrogen acceptor. The color intensity was measured at wavelength of 480 mμ with Beckman Model DU spectrophotometer.

The enzyme activity was expressed as mean formazan formed per mg. dry weight per 10 minutes ± standard deviation. The difference between control and test group was examined by statistical analysis.

SYNTHESIS OF MYCOLOYLAMINO ACID

The Sheehan's method for peptide synthesis (*10*) was applied to the preparation of mycoloylamino acid esters. Since all the preparations of the mycoloylamino acids were carried out in about the same way, the method for the synthesis of mycoloyl-L-leucine will be described in detail as an example.

To a solution of 0.65 g. (0.5×10^{-3} moles) of mycolic acid in 15 ml. of ether, freshly prepared L-leucine methylester* (0.10 g., 0.6×10^{-3} moles) and DCD (0.10 g., 0.5×10^{-3} moles) were added.

A precipitate of N, N′-dicyclohexylurea was formed within 20 to 30 minutes. After being left overnight at room temperature, the urea (about 0.10 g.) was removed by filtration and the filtrate was extracted three times with 0.5 *N* HCl under cooling and dried over Na_2SO_4. After subsequent removal of the solvent, 0.56 g. of a white powder was obtained.

To remove possible side products, the white powder dissolved in 30 ml. of petroleum ether was then applied to the column of alumina (activity 2, 10 g.) and eluted with petroleum ether, mixture of petroleum ether-benzene (9 : 1), benzene, ether and mixture of ether-acetic acid (9 : 1) successively.

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* Prepared by neutralizing an aqueous solution of L-leucine methylester hydrochloride to pH 8.0 with potassium carbonate and subsequent extraction into ether.

In the case of mycoloyl-L-leucine two fractions were obtained by this chromatographic separation, as shown in Table I.

TABLE I

Chromatographic Separation of Mycoloyl-L-leucine

Fraction	Eluted with 25 ml. of:	Yield
		mg.
0	Petroleum ether	0
1	Petroleum ether	0
2	Petroleum ether	0
3	Petroleum ether/benzene 9 : 1	0
4	Petroleum ether/benzene 9 : 1	0
5	Benzene	0
6	Benzene	trace
7	Ether	125
8	Ether	48
9	Ether	24
10	Ether	trace
11	Ether	0
12	Ether/acetic acid 10%	200
13	Ether/acetic acid 10%	65
14	Ether/acetic acid 10%	trace
15	Ether/acetic acid 10%	0
Recovered		426=83%

0.56 g. of crude mycoloyl-L-leucine was dissolved in 30 ml. of petroleum ether and adsorbed on 10 g. of alumina.

The first portion (fractions 7, 8, 9,) eluted by ether were combined and recrystallized from ether-methanol. Yield was 27 per cent based on mycolic acid. This substance was neutral to methyl red, ninhydrin negative and melted at 34–35°. After acid hydrolysis of this material leucine was detected by paper chromatography in an aqueous extract and from the other extract was obtained a white powder melting at 52°, which was identified as mycolic acid after two recrystallization from ether-methanol. The amount of leucine in this hydrolysate was roughly equivalent to that of mycolic acid, when measured chromatographically on Toyo-roshi No. 2 paper strips.

Analysis—Calcd. for $C_{94}H_{187}O_5N$ (Mycoloyl-L-leucine methylester):
C, 78.99; H, 12.89; N, 0.98.
Found: C, 79.66; H, 12.95; N, 1.42.

Infrared spectum (Fig. 1, No. 1); the spectrum showed absorptions at 3360, 2900, 2650, 1740, 1700, 1630, 1540, 1470, 1380, 1350, 1270, 1250, 1215, 1155, 1120, 1095, 1075, 1020, 965, 890, 720, cm^{-1}.

The second portions (fraction 12, 13) were similarly treated and gave a ninhydrin negative, acidic (methyl red) material, melting point 47–48°. Yield was 32 per cent based on mycolic acid. After acid hydrolysis mycolic acid and leucine roughly equivalent to mycolic acid were both detected in the same way mentioned above.

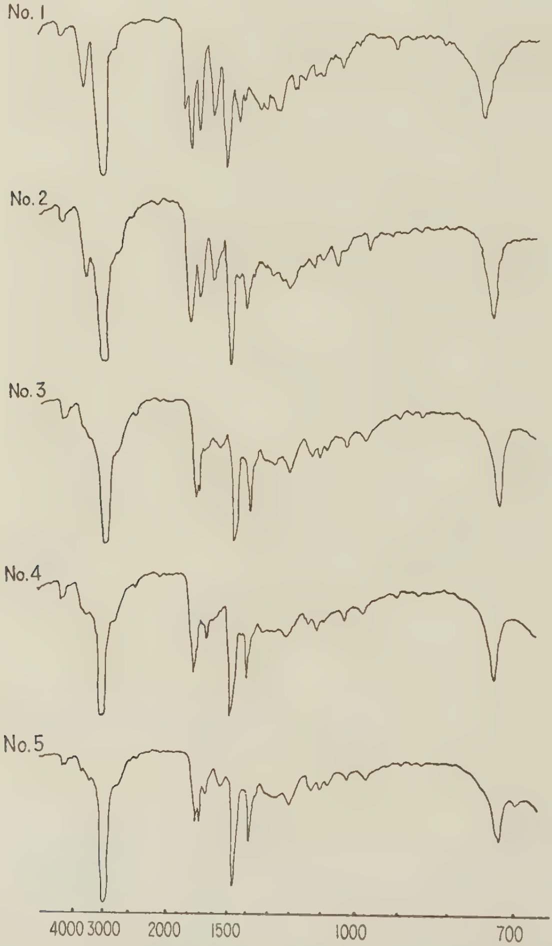

Fig. 1. Infrared spectrum of mycoloylamino acids.

No. 1. Mycoloyl-L-leucine methylester (Table I, fractions 7, 8, 9)
No. 2. Mycoloyl-L-leucine (Table I, fractions 12, 13)
No. 3. Mycoloyl-L-valine (Table II, fractions 11, 12)
No. 4. Mycoloyl-DL-alanine
No. 5. Mycoloyl-DL-phenylalanine

Analysis—Cald. for $C_{93}H_{185}O_5N$ (Mycoloyl-L-leucine):
C, 79.94; H, 13.25; N, 1.00.
Found: C, 79.57; H, 13.13; N, 1.24.

Infrared spectrum (Fig. 1, No. 2); the spectrum showed absorptions at 3350, 2900, 2650, 1700, 1640, 1545, 1480, 1380, 1275, 1235, 1205, 1155, 1130, 1095, 1015, 965, 885, 720, cm^{-1}.

Elementary analysis, the appearnce of -CO-NH-absorptions in the infrared spectrum and the detection of leucine and mycolic acid after acid hydrolysis indicate that above described two materials are the conjugate of mycolic acid and leucine and the conjugate of mycolic acid and methylester, respectively. Morever, the facts that leucine roughly equivalent to mycolic acid was detected upon acid hydrolysis and the rather higher nitrogen value than calculated was found in their elementary analysis suggest that they are practically free from unreacted mycolic acid. There are little differences between above two materials in their elementary analysis. However, the first material is (1) neutral, (2) melted at relatively low temperature, (3) eluted by ether alone and (4) its infrared spectrum showed a strong ester absorption at 1740, cm^{-1} and a very weak, broad absorption between 2700 and 2600 cm^{-1}, which can be assigned to -OH of carboxyl group. On the contrary, the second material is (1) acidic, (2) melted at higher temperature, (3) not eluted by ether alone but by ether containing acetic acid and (4) its infrared spectrum showed a stronger broad absorption between 2700 and 2600 cm^{-1} and the absence of an ester absorption at 1740 cm^{-1}. These data indicate that the first material is mycoloyl-L-leucine methylester and the second is mycoloyl-L-leuicne. Thus mycoloyl-L-leucine methylester seems to be rather unstable, being easily hydrolysed on the alumina column or by ordinary mild treatments. This unstability was repeatedly observed with other mycoloylamino acid esters. In this connection, it was stated by No. 11 that mycolic acid ester was also partially hydrolyzed on the silica gel column (*11*).

The other three conjugates: mycoloyl-DL-phenylalanine, mycoloyl-DL-valine and mycoloyl-L-alanine were prepared in the same way as in the case of mycoloyl-L-leucine. However, it was observed that in those cases considerable amounts of side products were formed. The side products were removed by the chromatography as shown in Tale II as is the case of mycoloyl-valine.

The first portions (fraction 0,1) in Table II eluted in the very beginning with petroleum ether were recrytallized from ether-methanol. Valine was not detected by paper chromatography upon acid hydrolysis. The combustion analysis (the Dumas' method) indicated the absence of nitrogen. Infrared spectrum showed the absorptions at 1800 and 1695 cm^{-1} which indicated the presence of acid anhydride. The above data seem to indicate that these portions are composed of a side product, mycolic acid anhydride.

The second portion (fraction 7) gave no ninhydrin positive spot on paper chromatogram after acid hydrolysis and showed high nitrogen value (1.65 per cent) in elementary analysis, suggesting that this is another side product, presumably mycoloyl-N, N'-dicyclohexylurea. Since these side products were not desired mycolic acid derivatives, no further confirmation was performed.

The third portions (fractions 11, 12) were similarly treated and gave a ninhydrin positive spot corresponding to valine upon hydrolysis. These portions were shown to be composed of mycoloyl-L-valine.

m.p. 52–53° Yield 19 per cent

Analysis—Calcd. for $C_{92}H_{183}O_5N$:

C, 79.94; H, 13.23; N, 1.01.

Found: C, 80.71; H, 12.84; N, 1.12.

Infrared spectrum (Fig. 1, No. 3): the spectrum showed absorptions at 2900, 2700, 1700, 1650, 1540, 1470, 1380, 1270, 1200, 1120, 1100, 1080, 1020, 970, 720, cm^{-1}.

TABLE II

Chromatographic Separation of Mycoloyl-DL-Valine

Fraction	Eluted with 50 ml.:	Yield
		mg.
0	Petroleum ether	158
1	Petroleum ether	162
2	Petroleum ether	trace
3	Petroleum ether/benzene 7 : 3	trace
4	Petroleum ether/benzene 7 : 3	14
5	Benzene	trace
6	Benzene	0
7	Ether	126
8	Ether	53
9	Ether	trace
10	Ether	0
11	Ether/acetic acid 10%	184
12	Ether/acetic acid 10%	18
13	Ether/acetic acid 10%	5
14	Ether/acetic acid 10%	trace
15	Ether/acetic acid 10%	0
Recovered		720=93%

0.774 g. of crude mycoloyl-DL-valine was dissolved in 40 ml. of petroleum ether and adsorbed on 11 g. of alumina.

Mycoloyl-DL-alanine and mycoloyl-DL-phenylalanine were prepared in the similar fashion, being accompanied by the same side products as in the case of mycoloyl-DL-valine.

Mycoloyl-DL-alanine

m.p. 51–52° Yield 22 per cent

Analysis—Calcd. for $C_{90}H_{179}O_5N$:

C, 79.82; H, 13.22; N, 1.04.

Found: C, 80.1 ; H, 13.0 ; N, 1.00.

Infrared spectrum (Fig. 1, No. 4): the spectrum showed absorptions at 3350, 2950, 2650, 1700, 1620, 1470, 1380, 1300, 1200, 1120, 1100, 1080, 1015, 965, 720, cm^{-1}.

Mycoloyl-DL-phenylalanine
m.p. 52-53° Yield 21 per cent

Analysis—Calcd. for $C_{96}H_{183}O_5N$:
C, 80.60; H, 12.80; N, 0.96.
Found: C, 80.70; H, 12.80; N, 1.0.

Infrared spectrum (Fig. 1, No. 5): the spectrum showed absorptions at 3350, 2950, 2700, 1720, 1690, 1640, 1540, 1470, 1380, 1270, 1210, 1130, 1100, 1080, 1020, 970, 720, 695, cm^{-1}.

BIOLOGICAL ACTIVITIES OF MYCOLOYLAMINO ACID

Toxicity of Synthesized Mycoloylamino Acids for Mice—All four synthesized mycoloylamino acids and free mycolic acid did not exert any cord factor-like toxicity for mice in terms of loss of body weight and death from the successive intraperitoneal injections of 0.1 mg.

Effect of Synthesized Mycoloylamino Acids on Succinic Dehydrogenase Activity of Mouse Liver—As shown in Table III, mycoloylleucine, mycoloylvaline and mycoloylalanine caused a significant decrease of succinic dehydrogenase activity of mice liver 24 hours after the intraperitoneal injection of 0.1 mg., while the same dosis of free mycolic acid and mycoloylphenylalanine were without effect.

Table III

Effect of Synthesized Mycoloylamino acids on Succinic Dehydrogenase Activity of Mouse Liver

	μg. Formazan form/mg. Dry Weight/10 min.[1]	
Test Materials	Control	Experimental
Mycoloylleucine	4.3±0.90	3.2±0.85[2]
Mycoloylvaline	4.6±0.45	3.3±0.90[2]
Mycoloylalanine	5.9±0.90	2.6±0.85[2]
Mycoloylphenylalanine	3.2±2.0	3.6±0.93
Mycolic acid	4.2±1.4	4.1±0.82

1) Expressed as average standard deviation of five animals. Control animals received 0.1 ml. olive oil only. Each mycoloylamino acids were injected into mice intraperitoneally at the dosis of 0.1 ml. olive oil, approximately 24 hrs. before the determination of enzyme activity.

2) Significant ($\alpha=0.001$).

DISCUSSION

It was suggested by Kato *et al.* that the basic mechanism of the toxicity of cord factor is the *in vivo* inhibition of liver succinic dehydrogenase system (*5*). The fact that some amino acid conjugates of mycolic acid also decrease the activity of the succinic dehydrogenase system of mouse liver seems to suggest that the determinant group of cord factor in its action on the succinic dehydrogenase system is the mycolic acid moiety of its structure. It seems rather controversial that synthesized mycoloylamino acids inhibit the succinic dehydrogenase system without causing any apparent toxicity for mice. There remains, however, the possibility that the moiety which conjugates with mycolic acid is responsible for the duration of the inhibition of this enzyme activity and the apperences of toxicity for mice. This point will be analyzed in further experiments.

SUMMARY

1. The conjugates of mycolic acid and several kinds of amino acid (leucine, valine, alanine, and phenylalanine) were synthesized.

2. The biological activities of the synthesized mycoloylamino acid were tested and it was shown that the activity of succinic dehydrogenase of mice liver was decreased 24 hours after the intraperitoneal injection of 0.1 mg. of mycoloylleucine, mycoloylvaline, and mycoloylalanine, while the same dosis of free mycolic acid and mycoloylphenylalanine were without effect.

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STUDIES ON THE C-TERMINAL AND ACIDIC AMINO ACID CONTAINING PEPTIDES OBTAINED FROM OVALBUMIN BY PARTIAL HYDRAZINOLYSIS

By ICHIRO HARUNA* AND SHIRO AKABORI

(From the Laboratory of Biochemistry, Faculty of Science,
Osaka University, Osaka)

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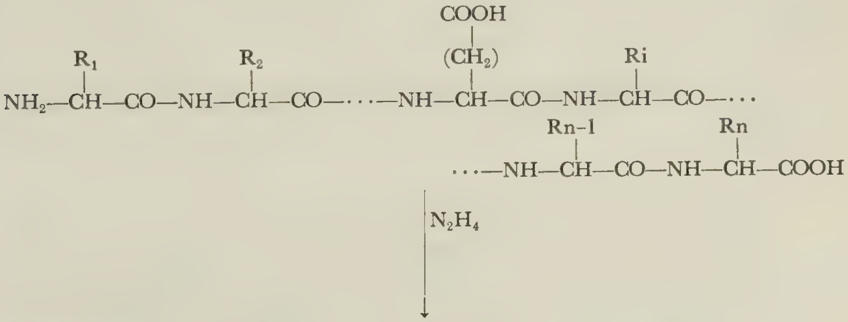
Akabori, Ohno and Narita (*1*) were the first to propose hydrazinolysis as a method for the characterization of the C-terminal amino acid residues of proteins and peptides. This method was later improved by introducing Sanger's dinitrophenylation technique for the separation of free amino acids from amino acid hydrazides produced in the hydrazinolysates (*2*). The usefulness of the hydrazinolysis method has since been widely recognized and the C-terminal residues of a variety of proteins have been analysed by this method with mostly satisfactory results (*3–12*).

In 1955, Ohno (*13*) and Niu and Fraenkel-Conrat (*14*, *15*) independently succeeded in extending the hydrazinolysis method to elucidate the amino acid sequence of the C-terminal region of proteins. The principle of the extended method consisted in the liberation of free, non-hydrazidated peptides from the C-terminal part of proteins on partial hydrazinolysis. These free peptides were then separated from the other peptide hydrazides and analysed for their sequence by the conventional methods.

In the present study it was intended to isolate not only the C-terminal peptides but also acidic amino acid containing peptide hydrazides from a partial hydrazinolysate of ovalbumin. The procedures employed for this purpose may be understandable from the following scheme:



$$NH_2{-}\overset{R_1}{\overset{|}{C}H}{-}CO{-}NH{-}\overset{R_2}{\overset{|}{C}H}{-}CO{-}\cdots{-}NH{-}\overset{\overset{\overset{COOH}{|}}{(CH_2)}}{\overset{|}{C}H}{-}CO{-}NH{-}\overset{Ri}{\overset{|}{C}H}{-}CO{-}\cdots$$

$$\cdots{-}NH{-}\overset{Rn\text{-}1}{\overset{|}{C}H}{-}CO{-}NH{-}\overset{Rn}{\overset{|}{C}H}{-}COOH$$

$$\Big\downarrow{\scriptstyle N_2H_4}$$

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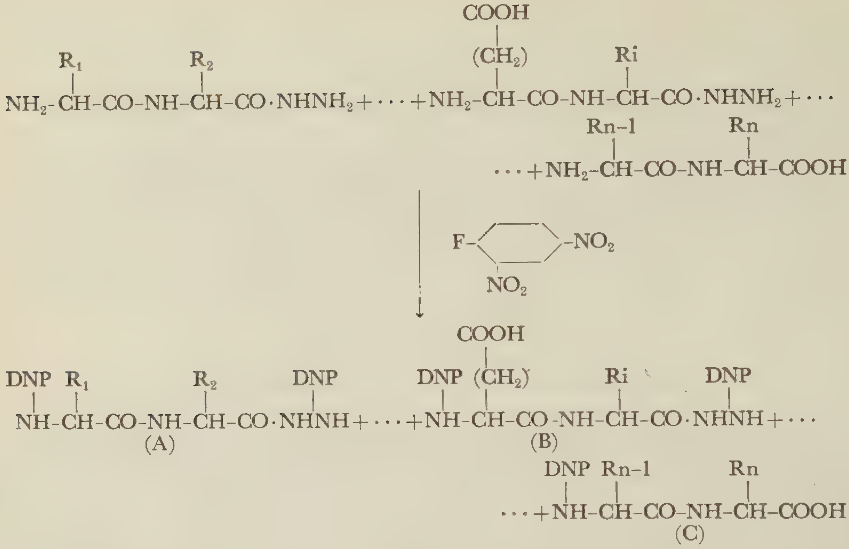
* Present address: Institute for Virus Research, Kyoto University, Kyoto.



When ovalbumin is subjected to partial hydrazinolysis, a mixture of peptide hydrazides derived from the non-C-terminal part and free peptides from the C-terminal region will be obtained. Dinitrophenylation of the partial hydrazinolysate with dinitrofluorobenzene will give rise to a mixture of di-DNP-peptide hydrazides (A, B *etc.*) and DNP-peptides (C). As can be seen from the scheme, acidic DNP derivatives will be formed both from the C-terminal peptide groups and from non-C-terminal peptide parts containing acidic amino acid residues. These acidic di-DNP-peptide derivatives (B) and DNP-peptides (C) can now be separated from other neutral peptide derivatives (such as A) by fractional extraction.

The acidic fraction thus obtained was fractionated by means of counter-current distribution, followed by column and paper chromatography, and 38 seemingly pure acidic DNP-peptide derivatives were obtained. The N-terminal residues and constituent amino acids of 35 out of the 38 DNP-compounds were analysed, and the structures of 5 dipeptides and 2 tripeptides were determined. It was thus found that the C-terminal part of ovalbumin has the sequence, Val-Ser-Pro, in confirmation of the work of Niu and Fraenkel-Conrat (*14*). It was also suggested that the peptide bond linking an amino acid residue to an acidic amino acid is quite susceptible to the action of hydrazine.

EXPERIMENTAL

Materials Ovalbumin—Ovalbumin was prepared either by the method of Cannan (*16*) or by that of Sϕrensen (*17*), recrystallized five times, dialyzed against distilled water and lyophilized.

Anhydrous Hydrazine—Anhydrous hydrazine was prepared by distilling 80 per cent hydrazine hydrate with calcium oxide according to Stähler (*18*). The preparation obtained was more than 99 per cent pure.

Hydrazinolysis—Twenty grams of ovalbumin were hydrazinolyzed with about 100 g. of anhydrous hydrazine for 3 hours at 100°. At this stage of hydrazinolysis, 0.3 mole of C-terminal amino acid appeared in the free state and the other C-terminal remained in peptide linkages.

Dinitrophenylation and Fractionation—The reaction mixture was evaporated *in vacuo* over sulfuric acid to remove excess hydrazine. The dinitrophenylation of the hydrazinolysate and the fractional extraction of the C-terminal and acidic amino acid containing DNP-peptides from other neutral DNP-peptide hydrazides were performed according to principally the same procedure as reported previously (*19*). In the present study, however the pre-treatment with aldehydes was avoided to preserve acidic amino acid containing peptide hydrazides. The partial hydrazinolysate was dissolved in 1 liter of water and added with 50 g. of sodium bicarbonate. Fifty grams of DNFB in 2 liters of ethanol were added to the solution and the mixture was shaken for 3 hours in a dark room. Most of the alcohol was removed by distillation under reduced pressure at room temperature. The solution was divided into three portions for the sake of convenience and each portion was fractionated as follows. Two hundreds milliliters of water were added to the solution and the mixture was acidified with 2 *N* hydrochloric acid. The mixture was then successively extracted with 300, 200 and 100 ml. of ethyl acetate to extract almost all the DNP-compounds. The ethyl acetate extracts were combined and further extracted seven times with each 200 ml. portion of 2 per cent sodium bicarbonate solution. The DNP-compounds bearing free carboxyl groups were extracted into the bicarbonate solution, leaving non-acidic DNP-compounds in the ethyl acetate layer. The bicarbonate solution was washed three times with each 200 ml. portion of ethyl acetate to prevent the contamination of neutral peptide hydrazides. The bicarbonate layer was acidified with 2 *N* hydrochloric acid and successively extracted with 200, 100 and 50 ml. of ethyl acetate. The ethyl acetate extracts were combined, evaporated *in vacuo* and the residue was subjected to fractionation.

Group Separation by Counter-Current Distribution—Column chromatography using either silica gel (*20*, *21*) or "Hyflo Supercel" (*22*) has so far usually been used for the separation of DNP-peptides. It has, however, recently been found that the counter-current distribution method is more suitable for the bulk separation of large amounts of DNP-peptide mixtures (*23*, *24*). Therefore, the fractionation of DNP-peptides was primarily carried out by counter-current distribution in this study.

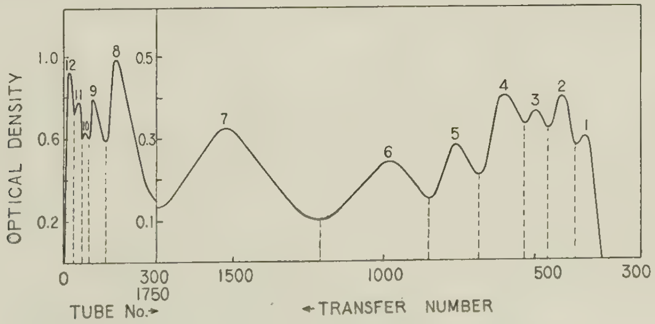


FIG. 1. Distribution pattern of partial hydrazinolysate.
Solvent: chloroform: acetic acid: 0.1 *N* HCl (2 : 2 : 1 *v/v*)

The solvent system used for the first preliminary distribution was prepared by equilibrating chloroform, glacial acetic acid and 0.1 N aqueous HCl (2 : 2 : 1 v/v). The phase volume in each distribution train was 10 ml. The distribution apparatus employed was a fully automatic type and had 295 tubes in total. The sample was placed in a bank of 5 tubes at the beginning. The treatment was carried out at room temperature in the dark. After completing 1455 transfers, the filling device was disconnected, no further upper phase was added, and the apparatus was driven until all the upper phases had migrated into the fraction collector. The last phase left the train at 1750 transfers. The distribution pattern shown in Fig. 1 was obtained by measuring optical absorption of each tube at 430 mμ. All cuts of the pattern were still complex mixtures. Therefore, further fractionation of each cut was performed by chromatography.

Chromatographic Purification—DNP-peptide fractions, which were obtained by the counter-current distribution method, were further purified by column chromatography using either silica gel or "Hyflo Supercel" and by paper chromatography. In some experiments elution chromatography using "Hyflo Supercel" was also used. "Hyflo Supercel" column chromatography was found to be suitable for the bulk separation of large amounts of DNP-peptide mixtures.

When a mixture of DNP-peptides was subjected to the column chromatography, several bands were separated on the column. Each band was cut off, eluted with acetone, evaporated *in vacuo* and further subjected to two-dimensional paper chromatography. Solvents used were *n*-butanol : 0.1 per cent ammonia (the organic layer of 1 : 1 parts by volume mixture) for the first dimension and 1.5 M phosphate buffer of pH 6.0 (1 M NaH_2PO_4 + 0.5 M Na_2HPO_4) for the second dimension.

It was very often found that a fraction, which was regarded as pure by repeated chromatography, was proved to be still heterogeneous, since two or more DNP-amino acids (N-terminal of the peptides) were detected from it after acid hydrolysis. In such a case, the fractions were further subjected to repeated chromatography until they could be considered pure in terms of only one DNP-amino acid liberation on hydrolysis.

Analyses of the Isolated DNP-Peptides

The analyses of pure DNP-peptides thus obtained were carried out as follows.

i) *Identification of N-Terminal Amino Acids*—In order to determine the N-terminal amino acid, each DNP-peptide was hydrolysed at 105° in a sealed tube with 6 N hydrochloric acid for 7 hours. The cooled hydrolysate was diluted with an equal amount of water and shaken three times with ethyl acetate in order to extract the N-terminal DNP-amino acid which was identified by two-dimensional paper chromatography as described above. The hydrolysis was stopped in shorter time than usual, since DNP-amino acid tended to decompose on prolonged hydrolysis.

ii) *Identification of Constituent Amino Acids*—The DNP-peptide to be examined was throughly hydrolysed at 105° in a sealed tube with 6 N hydrochloric acid for 24 hours. The hydrolysate was diluted with water and extracted with ethyl acetate to remove the N-terminal DNP-amino acid. The aqueous layer containing free amino acids, derived from the non-N-terminal part, was evaporated to dryness *in vacuo* and hydrochloric acid was thoroughly removed. The amino acids in the residue were identified by two-dimensional paper chromatography using *n*-butanol : methylethylketone : water : 17 N ammonia (5 : 3 : 1 : 1 v/v) as the solvent for the first dimension and *n*-butanol : glacial acetic acid : water (4 : 1 : 1 v/v) for the second dimension (*25*)*. In order to further confirm the amino acid composition, the residue was dissolved in sodium bicarbonate solution ($NaHCO_3$ 0.04 g./ml.) and two volumes of 5 per cent ethanolic solution of DNFB were added.

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* Proline on the chromatogram was detected by the isatine reaction.

After shaking for two hours, the reaction mixture was transferred to a separatory funnel with ten volumes of water, extracted with ether to remove excess DNFB, acidified with 2 N hydrochloric acid, and extracted with ethyl acetate. The solvent was removed *in vacuo* and from the residue dinitrophenol was sublimed at 100° below 15 mm-Hg with a cold finger containing a piece of dry ice. The nonvolatile residue was then dissolved in ethyl acetate and analysed by two-dimensional paper chromatography as described above. If water-soluble DNP-amino acids were present, the aqueous fraction was treated in the same manner as the organic layer. The solvent used for the paper chromatography was *n*-butanol : acetic acid : water (4 : 1 : 1 *v/v*). After eluting each spot with 1 per cent bicarbonate solution the amount of DNP-amino acid was spectrophotometrically estimated at 360 mμ. (DNP-proline was determined at 385 mμ). Molar ratio of amino acids in a peptide was calculated from the values of optical density.

iii) Identification of C-Terminal Amino Acid—Since it had been known that ovalbumin possesses proline as the sole C-terminal residue (*19*), proline-containing DNP-peptides were further hydrazinolysed at 100° for 8 hours in order to determine if they were really C-terminal peptides. After hydrozinolysing, the reaction mixture was dinitrophenylated, fractionated and chromatographed as usual. To make sure, it was checked that DNP-amino acid had absorption maximum at 385 mμ. If DNP-peptide is acidic amino acid-containing peptide, free amino acid is not liberated by hydrazinolysis.

RESULTS

The procedures described above were applied to each fraction which were separated by the counter-current distribution.

Fractionation and Analysis of Fraction 3—Fraction 3 was separated into three fractions, 3–1, 3–2 and 3–3 (R_f's 0.0, 0.29 and 1.0), by Celite column chromatography using *sec*-butanol : chloroform : M/10 phosphate buffer, pH

TABLE I

Analysis of Peptides Derived from Fraction 3

Peptide	3–1		3–2	
amino acid	optical density	molar ratio	optical density	molar ratio
N-terminal	Gly .035	1	Asp .038	1
Glu	.112	2	.048	1
Asp	.048	1	.044	1
Gly	.086	2	.066	2
Ser	.044	1	.046	1
Ala	.044	1	.042	1
Thr	.040	1	.034	1
Pro	.030	1	.044	1
Val	.080	2	.050	1
Leu & Ileu	.130	3	.084	2
Phe	.100	2	.084	2
His	.020	1	—	—

6.4 (20 : 80 : 100 *v/v*). Each band was eluted and its N-terminal amino acid identified. Glycine, serine and an acidic amino acid were detected as the N-terminal residues of Band 3–3 indicating that this fraction was still a mixture of peptides. Therefore, only Bands 3–1 and 3-2 were analysed for their constituent amino acids, and the results are shown in Table I. Because these two peptides contained proline as their constituent amino acid, they were both hydrazinolyzed in order to identify their C-terminal amino acids, but no free amino acid could be detected in the lyzates indicating that both peptides are acidic amino acid-containing peptide hydrazides.

Fractionation and Analysis of Fraction 4—Fraction 4 was dissolved in 1 per cent sodium bicarbonate solution, acidified with hydrochloric acid and extracted with ethyl acetate. A fraction (4H) was not extracted and remained in the aqueous layer. The ethyl acetate extract was then subjected to paper chromatography using *n*-butanol : 0.1 per cent ammonia (1 : 1 *v/v*) as the solvent. The R_f values of four fractions (4A, 4B, 4C and 4D) separated are 0.08, 0.32, 0.65 and 1.00, respectively. These fractions were further chromatographed with phosphate buffer. It can be seen that 4B and 4C were separated into three (4B-1, 4B-2 and 4B-3), and four components (4C-1, 4C-2, 4C-3 and 4C-4), respectively, while 4A and 4D appeared pure. Both 4B-1 and 4B-2 gave two fractions on rechromatography. The R_f values for each fraction are given in Table II. The N-terminal amino acids found for 4B-3 as well as 4C-3 were a mixture of an acidic amino acid, glycine and serine, a fact indicating their heterogeneity. 4A-1 and 4C-4 could not be analyzed because of their small quantity. The amino acid compositions of these peptides are recorded in Table III. Since all these peptides did not contain proline, the identification of C-terminal amino acid was not carried out. The constituent amino acids of 4D-1 were glutamic acid, glycine, alanine, serine and phenylalanine. But, because of their small quantity, the quantitative analysis was not performed.

TABLE II

R_f Values of Each Fraction on Paper Chromatogram

Solvent A		B					
Fraction	R_f	Fraction	R_f	Fraction	R_f	Fraction	R_f
4A	0.08	4A-1	0.62	4C-1	0.03	4D-1	0.19
4B	0.32	4B-1	0.03	4C-2	0.11	4B-1-1	0.0
4C	0.65	4B-2	0.12	4C-3	0.44	4B-1-2	0.04
4D	1.00	4B-3	0.35	4C-4	0.51	4B-2-1	0.10
						4B-2-2	0.13

Solvent A : *n*-butanol : 0.1% ammonia (1 : 1 *v/v*)
B : 1.5 *M* phosphate buffer, pH 6.0

Fractionation and Analysis of Fraction 5—Fraction 5 was fractionated by Celite column chromatography using *n*-butanol : chroloform : *M*/10 phosphate buffer (pH 6.4) (20 : 80 : 100 *v/v*) as the solvent. The R_f value of these five fractions, 5–1, 5–2, 5–3, 5–4 and 5–5, separated is 0.0, 0.23, 0.37, 0.48 and 1.00, respectively. Bands 5–2, 5–3 and 5–4 of the chromatogram were repeatedly rechromatographed with the same solvent. The analytical results for the N-terminal amino acids and the constituent amino acids of each of these peptides are presented in Table IV. The peptides 5–1 and 5–4 were not analyzed, since the analyses of hydrolysates showed that they were mixtures of peptides and did not possess proline in the constituent amino acids.

Table III

Analysis of Peptides Derived from Fraction 4

Peptide	4B-1-1		4B-1-2		4B-2-1		4B-2-2		4C-1		4C-2		4H	
Amino acid	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR
N-terminal	Asp .040	1	Glu .095	1	Asp .054	1	Asp .060	1	Asp .075	1	Asp .060	1	Gly .080	1
Glu	.080	2	.120	1	.068	1	.180	2	—		.072	1		
Asp	.088	2	.100	1	.069	1	.188	2	—		—		.326	3
Gly	—		—		.112	2	.064	1	—		.070	1	.086	1
Ser	.043	1	—		.078	1	.100	1	—		.072	1	.200	2
Ala	.088	2	.084	1	.062	1	.240	3	—		.074	1	.120	1
Thr	—		—		—		.084	1	—		—		.126	1
Val	.083	2	.102	1	—		—		—		.076	1	.104	1
Leu & Ileu	—		.098	1	.071	1	.164	2	—		—		.098	1
Phe	—		—		—		—		.103	1	.082	1	—	

OD: Optical Density.
MR: Molar Ratio.

Table IV

Analysis of Peptides Derived from Fraction 5

Peptide	5–2		5–3		5–4	
Amino acid	Optical density	Molar ratio	Optical density	Molar ratio	Optical density	Molar ratio
N-terminal	Asp .045	1	Ser .062	1	Ser .088	1
Ser	.058	1				
Ala			.078	1		
Glu			.072	1	.099	1

Fractionation and Analysis of Fraction 6—Fraction 6 was fractionated into four bands, 6–1, 6–2, 6–3 and 6–4 (R_f's 0.07, 0.20, 0.33 and 1.00, respectively), by Celite column chromatography. The solvent used was *n*-butanol: chloroform: *M*/10 phosphate buffer, pH 6.4 (20 : 80 : 100 *v/v*). Each band was repeatedly purified by the same chromatography and fractionated further by the following methods. Band 6–1 was separated into three fractions, 6–1–1, 6–1–2 and 6–1–3 (R_f's 0.0, 0.15 and 0.38, respectively), by DNP-paper chromatography using the system of 1.5 *M* phosphate buffer (pH 6.0). On a paper chromatogram with *n*-butanol : 0.1 per cent ammonia (1 : 1 *v/v*), three peptides were obtained from Band 6–1–2. But the analyses of these peptides were not accomplished because of their small quantities. For the same reason, Band 6–1–1 was not analyzed. The peptides 6–2 and 6–3 were separated further into two components by silica gel column chromatography using *n*-butanol : chloroform : acetic acid : water (3 : 97 : 5 : 95 *v/v*) as a solvent. The R_f values of peptides separated are given in Table V. The N-terminal amino acid of peptide 6–2–2 was aspartic acid, glutamic acid, glycine and alanine. The data of analysis of peptide 6–3–2 showed that this peptide contained glutamic acid as N-terminal and glutamic acid, proline and glycine as constituent amino acids. However the quantity of material was not

TABLE V

R_f Values of Peptides on Silica Gel Column Chromatogram

Solvent	A		B	
	Fraction	R_f	Fraction	R_f
	6–2–1	0.03	6–4–1	0.03
	6–2–2	0.28	6–4–2	0.19
	6–3–1	0.05	6–4–3	0.45
	6–3–2	0.43	6–4–4	1.00

Solvent A: *n*-butanol : chloroform : acetic acid : water (3 : 97 : 5 : 95 *v/v*)
B: chloroform : water (1 : 1 *v/v*)

TABLE VI

Analysis of Peptides Derived from Fraction 6

Peptide	6–1–3		6–2–1		6–3–1	
Amino acid	Optical density	Molar ratio	Optical density	Molar ratio	Optical density	Molar ratio
N-terminal	Asp .058	1	Val .148	1	Asp .093	1
Glu	.072	1				
Asp	.062	1				
Gly	.073	1			.085	1
Ser	.069	1	.168	1	.114	1
Ala	.074	1			.112	1
Val	.075	1			.129	1
Pro			.162	1		

sufficient for quantitative estimation. The results obtained from the analyses of other peptides are shown in Table VI. The data indicated that the peptide contained valine as N-terminal and serine and proline as constituent amino acids, each of which was equal in molar ratio. Since no acidic amino acids were detected in this peptide and proline was liberated on hydrazinolysis, this peptide must be derived from the C-terminal part of ovalbumin. This result coincides with that of Fraenkel-Conrat (*14*).

The fractionation and purification of 6–4 was performed by silica gel column chromatography. The solvent used was chloroform : water (1 : 1 *v/v*). Band 6–4 was fractionated into four components (Table V). These bands, 6–4–1, 6–4–2 and 6–4–3, were purified by rechromatography and hydrolyzed with hydrochloric acid for the characterization. However, in the present experiment, only qualitative results were obtained as shown in Table VII.

TABLE VII

Analysis of Peptides Derived from Fraction 6–4

Peptide	N-terminal	Amino acid
6–4–1	Asp or Glu	Gly, Ser, Ala, Pro, Val, Asp, Glu
6–4–2	Asp or Glu	Gly, Ala, Val, Ser, Pro, Asp, Glu
6–4–3	Asp or Glu	Asp, Glu, Gly, Ser, Ala, Val, Pro, Phe

Although the C-terminal amino acids could not be identified, these peptides might be C-terminal peptides, since proline, valine and serine were contained as constituent amino acids.

Fractionation and Analysis of Fraction 7—Fraction 7 was separated into four bands, 7A, 7B, 7C and 7D (R_f's 0.10, 0.40, 0.65 and 1.00, respectively), by Celite column chromatography using *n*-butanol : chloroform : $M/10$ phosphate buffer, pH 6.7, (10 : 90 : 100 *v/v*) as a solvent. Bands 7A, 7B, 7C and 7D were further separated by rechromatography with the same solvent (Table VIII).

TABLE VIII

R_f Values of Fractions Derived from 7A, 7B, 7C and 7D

Fraction	R_f	Fraction	R_f
7A-1	0.0	7B-1	0.20
7A-2	0.23	7B-2	0.44
7A-3	0.50	7C-1	0.33
		7D-1	1.00

The DNP-end group analyses of these separated bands elucidated that they were still mixtures of peptides. Therefore, the fractionation was repeated by chromatography. Band 7A-1 was separated by paper chromatography using phosphate buffer (pH 6.0, 1.5 M) as a solvent. Each band obtained was further separated by chromatography using *n*-butanol : 0.1 per cent ammonia (1 : 1 *v*/*v*) (Table IX). For the separation of 7B-1, 7B-2, 7C-1 and 7D-1, silica gel chromatography using the solvent system : *n*-butanol : chloroform : acetic acid : water (10 : 90 : 10 : 90 *v*/*v*) was successful. The R_f values for fractions separated are given in Table X. Bands separated from Fraction 7C by Celite column chromatography were eluted with acetone. When the solvent was evaporated, a crystalline material was obtained in quantity from this band. This material was dissolved in alcohol and then added with water for the recrystallization. The crystal showed a melting point of 112°. This fact, together with the other characters, indicated that this crystal was dinitrophenol. Band 7B-1-2 was separated into three fractions, 7B-1-2-1, 7B-1-2-2 and 7B-1-2-3 (R_f's 0.05, 0.30 and 0.50, respectively), by paper chromatography

TABLE IX

R_f Values of Fractions Derived from 7A-1 on Paper Chromatogram

Solvent	A		B	
	Fraction	R_f	Fraction	R_f
	7A-1-1	0.12	7A-1-1-1	0.39
			7A-1-1-2	1.00
	7A-1-2	0.27	7A-1-2-1	0.40
	7A-1-3	0.48	7A-1-3-1	0.05
			7A-1-3-2	0.35
	7A-1-4	0.64	7A-1-4-1	0.05

Solvent A : 1.5 *M* phosphate buffer (pH 6.0)
B : *n*-butanol : 0.1% ammonia (1 : 1 *v*/*v*)

TABLE X

R_f Values of Fractions Derived from 7B-1, 7B-2, 7C-1, and 7D-1 on Silica Gel Chromatogram

Fraction	R_f	Fraction	R_f
7B-1-1	0.09	7C-1-1	0.36
7B-1-2	0.32	7C-1-2	0.56
7B-1-3	0.72		
7B-2-1	0.0	7D-1-1	0.0
7B-2-2	0.26	7D-1-2	0.50
7B-2-3	0.54	7D-1-3	1.0
7B-2-4	1.0		

Solvent : *n*-butanol : chloroform : acetic acid ; water
(10 : 90 : 10 : 90 *v*/*v*)

using *n*-butanol : 0.1 per cent ammonia (1 : 1 *v/v*) as a solvent. Peptides separated as described above were hydrolyzed for the identification of their N-terminal residues and for the analyses of constituent amino acids. The results obtained up to now are shown in Table XI, XII and XIII. Since 7A-1-1-1 and 7A-1-3-2 contained proline as a constituent amino acid, the C-terminal analysis was carried out. The results, however, indicated that these peptides

TABLE XI

Analysis of Peptides Derived from Fraction 7A

Peptide	7A-1-1-1		7A-1-3-1		7A-1-3-2		7A-1-4-1		7A-2		7A-3	
Amino acid	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR
N-terminal	Asp .075	1	Asp .048	1	Glu .067	1	Glu .053	1	Asp .059	1	Glu .030	1
Glu	.106	1			.034	0			.081	1	.042	1
Asp	.021	0							.060	1		
Gly	.108	1			.080	1			.055	1	.034	1
Ser	.085	1			.070	1			.078	1	.036	1
Ala	.116	1	.062	1	.114	1	.070	1	.078	1	.036	1
Pro	.082	1			.065	1						
Leu & Ileu	.104	1										
Phe	.114	1										
Val	.114	1			.086	1			.063	1		

OD: Optical Density
MR: Molar Ratio

TABLE XII

Analysis of Peptides Derived from Fraction 7B

Peptide	7B-1-2-1		2B-1-2-2		7B-2-1		7B-2-2		7B-2-3		7B-2-4	
Amino acid	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR	OD	MR
N-terminal	Asp .049	1	Asp .040	1	Glu .047	1	Glu .068	1	Glu .040	1	Glu .030	1
Glu	.052	1			.055	1	.140	2	.018	0	.042	1
Asp	.016	0										
Gly	.056	1					.087	1			.037	1
Ser	.044	1			.094	2	.075	1	.047	1	.040	1
Ala	.064	1					.154	2			.040	1
Val					.109	2			.042	1		
Phe			.094	2								

OD: Optical Density
MR: Molar Ratio

were acidic amino acid containing peptide hydrazides, for no free C-terminal amino acid was released on hydrazinolysis.

TABLE XIII

Analysis of Peptides Derived from Fraction 7C

Peptide	7C-1-1		7C-1-2	
Amino acid	OD	MR	OD	MR
N-terminal	Tyr .025	1	Glu .020	1
Glu	.115	2		
Asp	.052	1		
Gly	.051	1	.028	1
Ser	.060	1	.031	1
Ala	.058	1		
Phe			.033	1

OD: Optical Density
MR: Molar Ratio

DISCUSSION

The experimental results described above may be summarized as recorded in Tables XIV and XV. In total, 35 DNP-peptides either derived from the C-terminal region or containing acidic amino acid residues were sufficiently purified from the partial hydrazinolysate of ovalbumin. Among these, 7 peptides listed in Table XIV were determined of their amino acid sequence. They are all di- or tripeptides. The other 28 peptides were analysed for their N-terminal residues and for their amino acid composition; the results of these analyses being presented in Table XV. In these tables, small figures added to the right side of the amino acid symbols indicate the content of the acids on a molar basis. Where no such figures are given, it should be understood that any quantitative data could not be obtained for the peptide in question.

It has been shown that proline is the sole C-terminal residue of intact ovalbumin (*14*, *19*). The peptides containing proline as their constituent amino acid were, therefore, carefully examined if they were really derived from the C-termini of the original protein. Thus it was established that only one peptide (6-2-1) having the sequence Val-Ser-Pro definitely represents one of the C-termini of ovalbumin. Although two other peptides (6–4–2 and 6–4–3) both containing proline, serine, and valine in addition to several other amino acids also appear to have been derived from the C-termini, the data are still insufficient to permit decisive conclusions. The C-terminal sequence Val-Ser-Pro has also been proposed by Niu and Fraenkel-Conrat (*14*) for ovalbumin.

A glance at the data recorded in Tables XIV and XV will immediately reveal a very interesting fact that out of 35 peptides examined as many as 28 peptides possess acidic amino acids as their N-terminal residues. It will be seen that 15 peptides have aspartic acid as their N-terminal residues, 10 glutamic acid, 3 aspartic acid or glutamic acid, 3 glycine, 2 serine, 1 valine, and 1 tyrosine. This fact strongly suggests that the peptide bonds at the N-site of acidic amino acids are more easily susceptible to the action of

TABLE XIV

Analysis of Peptide

No.	N-terminal	Amino acid composition	Original peptide
4C-1	Asp	Phe	Asp-Phe
5-2	Asp	Ser	Asp-Ser
5-4	Ser	Glu	Ser-Glu
6-2-1	Val	Ser$_1$, Pro$_1$	Val-Ser-Pro
7A-1-3-1	Asp	Ala	Asp-Ala
7A-1-4-1	Glu	Ala	Glu-Ala
7B-1-2-2	Asp	Phe$_2$	Asp-Phe-Phe

TABLE XV

Analysis of Peptide

No.	N-terminal	Amino acid composition
3-1	Gly	Glu$_2$, Asp$_1$, Gly$_2$, Ser$_1$, Ala$_1$, Thr$_1$, Pro$_1$, Val$_2$, Leu$_3$, Phe$_2$, His$_1$
3-2	Asp	Glu$_1$, Asp$_1$, Gly$_2$, Ser$_1$, Ala$_1$, Thr$_1$, Pro$_1$, Val$_1$, Leu$_2$, Phe$_2$
4H	Gly	Ala$_1$, Thr$_1$, Ser$_2$, Gly$_1$, Leu$_1$, Val$_1$, Asp or Glu$_3$
4B-1-1	Asp	Asp$_2$, Glu$_2$, Ser$_1$, Ala$_2$, Val$_2$
4B-1-2	Glu	Asp$_1$, Glu$_1$, Ala$_1$, Val$_1$, Leu$_1$
4B-2-1	Asp	Asp$_1$, Glu$_1$, Gly$_2$, Ser$_1$, Ala$_1$, Leu$_1$
4B-2-2	Asp	Asp$_2$, Glu$_2$, Gly$_1$, Ser$_1$, Ala$_3$, Thr$_1$, Leu$_2$
4C-2	Asp	Glu$_1$, Gly$_1$, Ser$_1$, Ala$_1$, Val$_1$, Phe$_1$
4D-1	Gly	Glu, Gly, Ala, Ser, Phe
5-3	Ser	Ala$_1$, Glu$_1$
6-1-3	Asp	Glu$_1$, Asp$_1$, Gly$_1$, Ser$_1$, Ala$_1$, Val$_1$
6-2-2	Asp	Asp, Glu, Gly, Ala
6-3-1	Asp	Gly$_1$, Ser$_1$, Ala$_1$, Val$_1$
6-3-2	Glu	Glu, Pro, Gly
6-4-1	Asp or Glu	Gly, Ser, Ala, Pro, Val, Asp, Glu
6-4-2	Asp or Glu	Gly, Ala, Val, Ser, Pro, Asp, Glu
6-4-3	Asp or Glu	Asp, Glu, Gly, Ser, Ala, Val, Pro, Phe
7A-1-1-1	Asp	Gly$_1$, Glu$_1$, Ala$_1$, Leu$_1$, Phe$_1$, Val$_1$, Ser$_1$, Pro$_1$
7A-1-3-2	Glu	Gly$_1$, Ser$_1$, Ala$_1$, Val$_1$, Pro$_1$
7A-2	Asp	Glu$_1$, Asp$_1$, Gly$_1$, Ser$_1$, Ala$_1$, Val$_1$
7A-3	Glu	Gly$_1$, Ser$_1$, Ala$_1$, Glu$_1$
7B-1-2-1	Asp	Gly$_1$, Ser$_1$, Ala$_1$, Glu$_1$
7B-2-1	Glu	Ser$_2$, Val$_2$, Glu$_1$
7B-2-2	Glu	Glu$_2$, Gly$_1$, Ser$_1$, Ala$_2$
7B-2-3	Glu	Ser$_1$, Val$_1$
7B-2-4	Glu	Gly$_1$, Ser$_1$, Ala$_1$, Glu$_1$
7C-1-1	Tyr	Glu$_2$, Asp$_1$, Gly$_1$, Ser$_1$, Ala$_1$
7C-1-2	Glu	Gly$_1$, Ser$_1$, Phe$_1$

hydrazine than the other peptide linkages.

Ovalbumin has been shown to be devoid of N-terminal groups as judged from the DNP method (*26*, *27*). Concerning the C-terminal residue of the protein, Steinberg (*28*) reported the liberation of alanine by the action of carboxypeptidase. However, he has later found that this was due to a DFP-sensitive enzyme contaminated in the preparation (*29*). Locker (*4*) also showed that the protein is not attacked by DFP-treated carboxypeptidase. Turner and Schmerzler (*30*), on the other hand, claimed that the C-terminal residue of ovalbumin is alanine on the basis of the results obtained by the thiohydantoin method. The present study, together with the work by Nui and Fraenkel-Conrat (*14*), has clearly indicated that the C-terminal region has the sequence Val-Ser-Pro. In view of the fact that proline residue situated at the C-termini of proteins and peptides can not be liberated by either carboxypeptidase or the thiohydantoin method, it seems that something must have been wrong in those studies in which alanine was identified as the C-terminal group.

Ottesen (*31*) has reported that ovalbumin has a closed ring structure with no N- and C-terminal residues and explained the transformations of the egg protein into plakalbumin on this basis. Since his results are radically different from those reported in the present paper, it seems still desirable to perform further critical experiments before one can draw a decisive conclusions concerning the C-terminal structure of ovalbumin.

SUMMARY

1. A procedure for the determination of C-terminal peptides and acidic amino acid containing peptides of proteins by means of partial hydrazinolysis was described.

2. Thirty five acidic DNP-peptide derivatives were isolated from a partial hydrazinolysate of ovalbumin and their N-terminal residues and amino acid compositions were analysed.

3. That the C-terminal part of the protein has the sequence, Val-Ser-Pro, was confirmed.

4. It was suggested that the peptide chain in protein is apt to be split at the N-sites of acidic amino acids by hydrazinolysis.

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AN IMMUNOCHEMICAL STUDY OF MICROBIAL AMYLASE. III.

By TSURU WADA

(*From the Institute of Applied Microbiology,*
University of Tokyo, Tokyo)

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In the previous papers the author reported on the preparation of antisera against various mold and bacterial amylases, the inhibition of amylase activity by these antisera, and the competitive inhibition of the action of antisera by the substrate, starch, and its hydrolysed products (*1–3*).

In the present study, antiserum against *p*-phenylazobenzoyl-Taka-amylase (*4*) was prepared. The inhibition of amylase- and maltosidase-activities of this PAB-TAA* and protease-modified PAB-TAA (*5*) by the anti-PAB-TAA and the protective effect of PAB on its enzyme activity from this inhibition were also studied.

METHODS

PAB-TAA—Crystalline PAB-TAA was prepared from TAA and PAB chloride according to the method of Ikenaka (*4*). It contained about one PAB residue per molecule.

M-PAB-TAA—Crystalline protease-modified M-PAB-TAA was kindly supplied by Miss. H. Toda of Chemistry Dept., Faculty of Science of the Osaka University** (*5*). This M-PAB-TAA preparation was homogeneous, from the observations by electrophoresis and ultracentrifuge.

Method for the Preparation of Anti-PAB-TAA Serum—Antigen: PAB-TAA was used as 15 mg. per ml. solution in physiological saline.

Antibody. Three rabbits weighing 3 Kg. or more were injected intravenously with 1 ml. of the above mentioned antigen solution.

Six injection were given, one every other day, in the course of 11 days. The rabbits were sacrified by bleeding from carotid artery 16 days after the last injection and sera were separated. The sera were treated at 56° for 30 minutes and stored in frozen state.

Assay of Maltosidase Activity—To 1 ml. of $N/5$ acetate buffer pH 5.3 was added 1 ml. of 0.5 per cent α-phenylmaltoside and 1 ml. of enzyme and incubated at 30° for 1 hour. Enzymic reaction was stopped by addition of 0.8 ml. of 20 per cent trichloroacetic acid and the reaction mixture was centrifuged after 30 minutes in the cold. After adjusting pH to 5.3 by addition of *N* NaOH, 1 ml. of 15 per cent Na_2CO_3 and 1 ml. of Folin phenol reagent were added to 3.5 ml. of supernatant solution, and the mixture was incubated at 30° for 20 minutes, and the intensity of the colour was determined spectrophotometrically at 660 mμ.

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* The abbreviations used throughout this paper include TAA, Taka-amylase A; PAB, *p*-phenylazobenzoic acid or *p*-phenylazobenzoyl; PAB-TAA, *p*-phenylazobenzoyl-Taka-amylase A; M-PAB-TAA, protease-modified *p*-phenylazobenzoyl-Taka-amylase A.

** According to a private communication from Miss. Toda, M-PAB-TAA was prepared by fractionation of a digest of PAB-TAA with *Streptomyces* protease for 6 days at 30°.

Methods for the preparation of anti-TAA serum and the assay of amylase activity were described in the previous papers (*1-3*).

Serological properties of antisera were studied by gel diffusion method, ring test, and immunoelectrophoresis (*3*).

RESULTS

Serological Observation—Ring test was performed using serial 2 folds dilution of anti-PAB-TAA and 150 μg. per ml. PAB-TAA solution as shown in Table I-(a). The reaction between these sera and TAA solution was shown in

TABLE I

(a) *Precipitation Reaction of Anti-PAB-TAA Serum and PAB-TAA*1)

Batch No. of anti-PAB-TAA serum	Serum dilution 1×2^n, n=										
	0	1	2	3	4	5	6	7	8	9	10
785	3	3	3	2	2	2	1	0	0	0	0
786	3	3	3	3	2	2	2	2	1	1	0
787	2	2	2	1	1	1	0	0	0	0	0

1) Results of ring test after 1 hour.
150 μg./ml. PAB-TAA solution was used as antigen.

(b) *Precipitation Reaction of Anti-PAB-TAA Serum and TAA*2)

Batch No. of anti-PAB-TAA serum	Serum dilution 1×2^n, n=										
	0	1	2	3	4	5	6	7	8	9	10
785	3	3	3	2	2	1	1	0	0	0	0
786	3	3	3	3	2	2	2	1	1	0	0
787	2	2	2	1	0	0	0	0	0	0	0

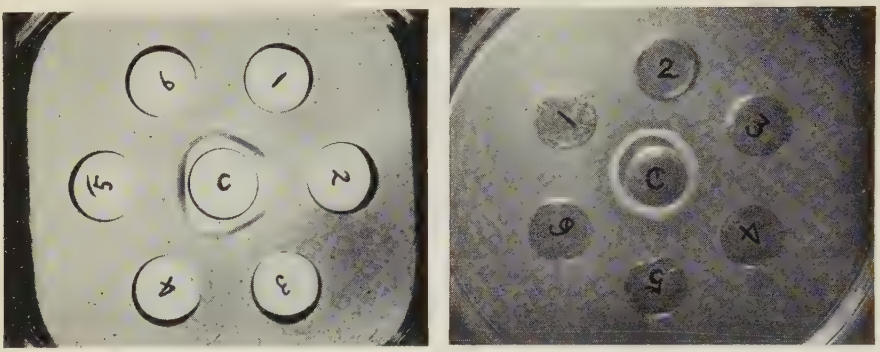
2) Results of ring test after 1 hour.
200 μg./ml. TAA solution was used as antigen.

(c) *Precipitation Reaction of Anti-TAA Serum and PAB-TAA*3)

Batch No. of anti-TAA serum	Serum dilution 1×2^n, n=										
	0	1	2	3	4	5	6	7	8	9	10
69	3	3	3	2	2	1	1	1	0	0	0
70	2	2	2	1	1	0	0	0	0	0	0

3) Results of ring test after 1 hour.
150 μg./ml. PAB-TAA solution was used as antigen.

Table I-(b). The reaction between PAB-TAA solution and anti-TAA serum or anti-PAB-TAA serum was shown in table I-(c). Precipitation reaction



(photograph 1) (photograph 2)

Fig. 1. Precipitation reactions between TAA, PAB-TAA, and M-PAB-TAA and PAB-TAA- and TAA-antisera according to the gel diffusion method.

Basin 1. TAA, 1 mg. per ml.
,, 2. TAA, 0.5 mg. per ml.
,, 3. PAB-TAA, ca. 1 mg. per ml.
,, 4. PAB-TAA, ca. 0.2 mg. per ml.
,, 5. M-PAB-TAA, ca. 1 mg. per ml.
,, 6. M-PAB-TAA, ca. 0.2 mg. per ml.
Center: anti-PAB-TAA (photograph 1) or anti-TAA (photograph 2)

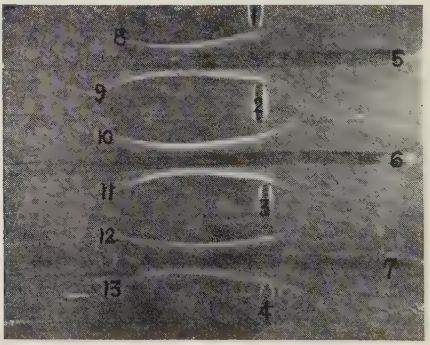


Fig. 2. Immunoelectrophoresis of PAB-TAA (by Grabar's method).
Medium: 2% agar in pH 8.4, 1/10 *M* barbitar buffer.
Antigen: 15 mg./ml. PAB-TAA added in basins 1 and 2.
30 mg./ml. TAA added in basins 3 and 4.

Electrophoresis was carried out at 250 volts, 8 mA. for 3 hours. After electrophoresis anti-PAB-TAA serum No. 68, 69 were added to basins 5 and 6, and anti-TAA serum No. 70 to basin 7. Precipitation line between PAB-TAA and its antiserum on the anodic side was 8, 9, and 10, precipitation line between TAA and its antiserum on the anodic side was 13 and 12, and precipitation line between TAA and anti-PAB-TAA serum on the anodic side was 11.

according to the gel diffusion method between TAA, PAB-TAA and M-PAB-TAA and PAB-TAA antiserum or TAA antiserum were shown in Fig. 1. One precipitation line was observed between PAB-TAA antiserum and TAA, PAB-TAA and M-PAB-TAA, and also between TAA antiserum and TAA, PAB-TAA, M-PAB-TAA, respectively.

PAB-TAA antiserum was examined immunoelectrophoretically. Results were shown in Fig. 2.

Inhibition of Enzymatic Activity of PAB-TAA and M-PAB-TAA by Anti-PAB-TAA Serum—The inhibition of amylase- and maltosidase-activities of PAB-TAA by anti-PAB-TAA serum and by anti-TAA serum was examined. The inhibition of amylase activity of TAA by anti-PAB-TAA serum was also

TABLE II

(a) *Inhibition of Maltosidase Activity of PAB-TAA and M-PAB-TAA by Anti-PAB-TAA Serum*

Anti-PAB-TAA serum	Maltosidase activity (O. D. at 660 mμ)	
	M-PAB-TAA	PAB-TAA
0.0 ml.	0.335	0.365
0.1 ml.	0.295	0.340
0.2 ml.	0.270	0.340
0.4 ml.	0.245	0.280
0.6 ml.	0.210	0.265
0.8 ml.	0.220	

Reaction mixture contained 0.4 ml. of M-PAB-TAA (30 μg./ml.) or PAB-TAA (40 μg./ml.) solution and PAB-TAA antiserum (10 fold dilution) in a final volume of 1.2 ml. After incubation of 60 minutes at 30°, enzyme activity was assayed by determining phenol formed from *p*-phenyl maltoside, using Folin phenol reagent (*4*).

(b) *Inhibition of Maltosidase Activity of PAB-TAA and M-PAB-TAA by Anti-TAA Serum.*

Anti-TAA serum	Maltosidase activity (O. D. at 660 mμ)	
	M-PAB-TAA	PAB-TAA
0.0 ml.	0.430	0.540
0.1 ml.	0.380	0.495
0.2 ml.	0.320	0.460
0.4 ml.	0.340	0.430
0.6 ml.	0.340	0.340

Reaction mixture contained 0.4 ml. of M-PAB-TAA (40 μg./ml.) or PAB-TAA (50 μg./ml.) solution and TAA antiserum (10 fold dilution) in a final volume of 1.2 ml. After incubation of 60 minutes at 30°, enzyme activity was assayed as described in Table II (a).

studied. As shown in Table II, the results were essentially similar in the cases of inhibition of amylase and maltosidase activities.

The inhibition of amylase and maltosidase activities of M-PAB-TAA by anti PAB-TAA and anti-TAA was examined. As shown in Fig. 3 and Table II, the results were essentially similar in the case of inhibition of enzymatic activity of PAB-TAA by anti-PAB-TAA and anti-TAA antibody. In agree-

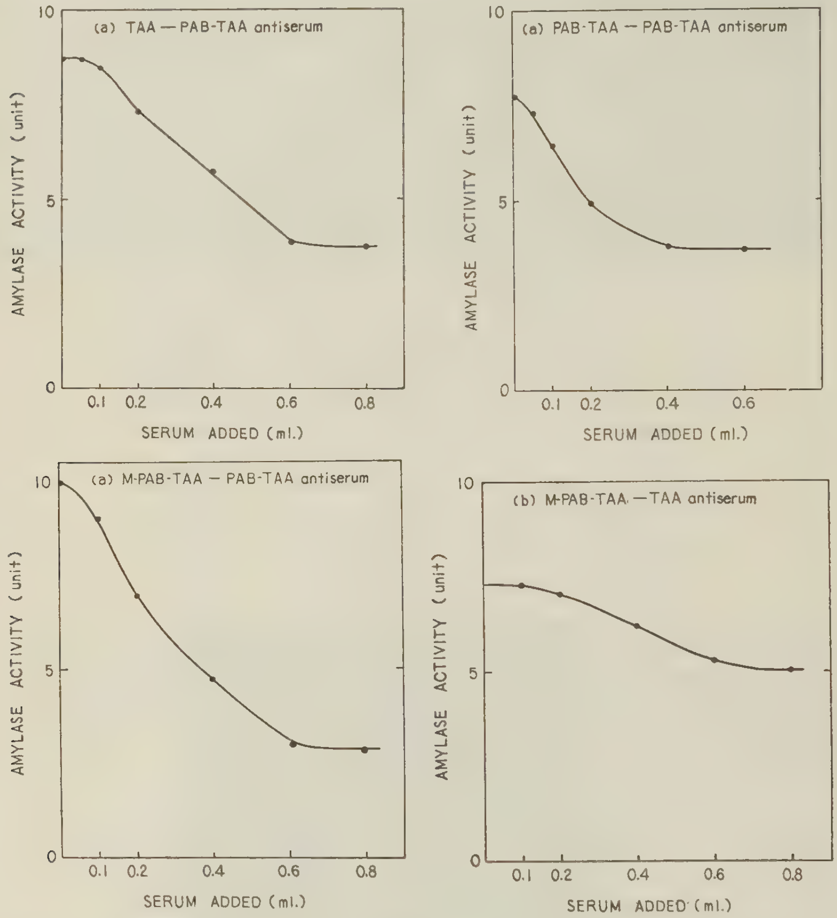


Fig. 3. Inhibition of amylase activity.

(a) Inhibition curve of amylase activity of TAA, PAB-TAA, and M-PAB-TAA by anti-PAB-TAA serum.

0.4 ml. of TAA (8.7 unit), 0.4 ml. of PAB-TAA (50 μg./ml.), or 0.5 ml. of M-PAB-TAA (40 μg./ml.) solution was incubated with PAB-TAA antiserum (10 fold dilution) at 40°. After 60 minutes, enzyme activity was assayed.

(b) Inhibition curve of amylase activity of M-PAB-TAA by anti-TAA serum. 0.5 ml. of M-PAB-TAA (ca. 40 μg./ml.) solution was incubated with anti-TAA serum (10 fold dilution) at 40°. After 60 minutes, enzyme activity was assayed.

ment with the results by the ring test and gel diffusion method, these results also suggest that there are cross reactions between TAA, PAB-TAA and M-PAB-TAA.

Effect of PAB and PAB-serine on the Reactions between PAB-TAA or TAA and Anti-PAB-TAA—Inhibition of the reactions between PAB-TAA and M-PAB-TAA, and anti-PAB-TAA by PAB was demonstrated by the protection of amylase and maltosidase activities of PAB-TAA and M-PAB-TAA from the inhibition by anti-PAB-TAA, and inhibition of PAB-TAA-anti-PAB-TAA

TABLE III

Effect of PAB and PAB-serine on the Enzymatic Neutralization Reaction of PAB-TAA—Anti-PAB-TAA Serum, M-PAB-TAA—Anti-PAB-TAA Serum, PAB-TAA—Anti-TAA Serum, and M-PAB-TAA—Anti-TAA Serum.

(a) *Effect on the Maltosidase Activity* (*Antigen: PAB-TAA and M-PAB-TAA*)

Antigen	Antiserum	Reagent	Maltosidase activity (O. D. at 660 mμ)	
	ml.	μg.	PAB added[1]	PAB-serine added
PAB-TAA	Anti-PAB-TAA			
	0.05	0	0.110	0.430
	0.05	5	0.150	0.440
	0.05	10	0.160	0.465
	0.05	20	0.185	0.490
	0.00	0	0.195	0.524

0.5 ml. of 10 fold dilution antiserum and 5, 10, 20 μg. of PAB or PAB-serine were mixed. After adjustment of pH to 6.2 (1) or 5.3, 0.5 ml. of 75 μg./ml. PAB-TAA was added. Maltosidase activity was assayed after the incubation for 1 hour at 30°.

Antigen	Antiserum ml.	PAB added μg.	Maltosidase activity (O. D. at 660 mμ)
M-PAB-TAA	Anti PAB-TAA		
	0.6	0	0.300
	0.6	100	0.415
M-PAB-TAA	Anti TAA		
	0.6	0	0.372
	0.6	100	0.380
M-PAB-TAA	0.85% saline solution		
	0.6	0	0.440
	0.6	100	0.440

0.6 ml. of antiserum (10 fold dilution) and 0.1 ml. of PAB (1 mg./ml.) was mixed. After the incubation of 1 hour at 40°, 0.5 ml. of M-PAB-TAA (70 μg./ml.) was added, adjusted pH to 6.2, total volume to 1.2 ml. with 0.85% saline solution. Maltosidase activity was assayed after the incubation of 1 hour at 40°.

reaction by PAB and PAB-serine was also demonstrated by the protection of precipitation by antigen-antibody reaction. The results were summarized in Table III. It was also shown that both PAB-serine had no effect on the reactions with anti-TAA serum. These results indicate a characteristic difference between the two antisera.

(b) *Effect on the Amylase Activity* (*Antigen*: *M-PAB-TAA*)

Antigen	Antiserum ml.	PAB-added μg.	Amylase activity unit
M-PAB-TAA	Anti-PAB-TAA		
	0.6	0	2.56
	0.6	100	4.6
M-PAB-TAA	Anti-TAA		
	0.6	0	6.4
	0.6	100	6.4
M-PAB-TAA	0.85% saline solution		
	0.6	0	11.2
	0.6	100	11.2

0.6 ml. of antiserum (5 fold dilution) and 0.1 ml. of PAB (1 mg./ml.) was mixed. After the incubation of 1 hour at 40°, 0.5 ml. of M-PAB-TAA (70 μg./ml.) was added and total volume was adjusted to 1.2 ml. with 0.85% saline solution. Amylase activity was asssayed after the incubation of 1 hour at 40°.

(c) *On the Precipitation Reacticn* (*Antigen*: *PAB-TAA*)

Reagent μg.	Amount of precipitation (O. D. at 660 mμ)	
	PAB added	PAB-serine added
0	0.440	0.270
25	0.440	0.270
50	0.420	0.260
100	0.180	0.180
200	0.180	0.150

The amount of anti-PAB-TAA added to PAB-TAA was the same in the ratio as that of anti-TAA added to TAA, which made maximum precitation. 0.4 ml. of antiserum and 25, 50, 100, 200 μg./ml. After 30 minutes, 0.5 ml. of 150 μg./ml. PAB or PAB-serine were mixed. PAB-TAA was added to these mixtures. After allowed to stand overnight at 3°, the precipitates were collected and their amounts were determined according to the method of Lawry *et al.* as described in the previous paper (*2*).

DISCUSSION

The foundation for the concept that the immunological specificity of antigens is intimately related to their chemical structure was laid by the work of Landsteiner, who coupled various chemical substituents to proteins and showed that antibodies produced against these artificial conjugated proteins exhibited reactions specific for the introduced groups. From this point of view, extensive immunochemical studies were undertaken by Pauling *et al.* and later numerous examples of such inhibition reaction have been described by many workers (*8–11*). On the other hand, systematic studies have been carried out in the Lab. of Akabori on the variation of relative activities of TAA accompanied with its chemical and enzymic modifications. Ikenaka reported a remarkable increase of the ratio, maltosidase activity to amylase activity, by introducing one PAB group into TAA and suggested that PAB group might be attached very close to the active site of TAA. Toda obtained enzymically modified PAB-TAA in crystalline state and confirmed that it was still enzymically active despite of a considerable change in the protein structure.

In the present investigation the author demonstrated the formation of a specific antibody against PAB-TAA, and it was found that an apparent antigen-antibody reaction between M-PAB-TAA and anti-PAB-TAA or anti-TAA occurred, as well as between PAB-TAA and anti-PAB-TAA or anti-TAA and between TAA and anti-TAA or anti-PAB-TAA. This fact showes that whole protein molecule is not necessary for the antigen-antibody reaction, *i.e.*, the part of PAB-TAA protein splitted off by the protease plays not important role in the immune reaction.

On the other hand PAB residue and enzymically active sites of PAB-TAA and M-PAB-TAA seem to be involved in the combination between M-PAB-TAA and anti-PAB-TAA, and between PAB-TAA and anti-PAB-TAA, because neutralization of enzymic activity of M-PAB-TAA and PAB-TAA is resulted by the addition of the antisera and the precipitation reactions are greatly affected by an addition of azo-compound, PAB. Both precipitation reaction between M-PAB-TAA or PAB-TAA and anti-TAA and neutralization of enzymic activity of M-PAB-TAA by anti-TAA sera were demonstrated but no effect of PAB against this neutralization reaction was observed.

Therefore, it appears that the active sites of the enzyme and the azo residues on the enzyme molecule, PAB-TAA or M-PAB-TAA, are located enough closely to be covered with a single molecule of antibody.

In the study on the active sites of TAA, Ikenaka found that PAB-TAA had a much less amylase activity but had rather higher maltosidase activity than that of original TAA, and he concluded that the enzymically active sites and azo residue located closely to each other on the enzyme molecule (*4*). The results described in the present paper are consistent with his view.

It is interesting to note that Porter reported an isolation of a small fragment of bovine serum albumin (BSA), which was obtained by chymotryptic digestion of BSA and could be dialyzed through the membrane but still retained the ability to react with anti-BSA (*12*).

In conclusion, it may be emphasized that the immunochemical technique could be, in certain cases, successfully applied to the study of active site of an enzyme protein.

SUMMARY

1. *p*-Phenylazobenzoyl Taka-amylase A is antigenic and gives rise to antisera when injected into rabbits in repeated doses.

2. It was demonstrated from the serological studies of antisera that there is an apparent cross reaction between Taka-amylase A and *p*-phenylazobenzoyl Taka-amylase and protease-modified *p*-phenylazobenzoyl Taka-amylase A.

3. Amylase- and maltosidase-activities of PAB-TAA and M-PAB-TAA were inhibited by antisera against PAB-TAA and TAA.

4. It was found that PAB protected PAB-TAA and M-PAB-TAA from the inhibition by its antiserum but no protection of PAB against the neutralization by anti-TAA was observed.

5. The results were consistent with the suggestion of Ikenaka that the enzymatically active sites and PAB-residue located close together on the enzyme molecule.

The author is indebted to Prof. S. Akabori of the Institute for Protein Research, Osaka University and to Prof. B. Maruo and Dr. H. Takahashi, the Institute of Applied Microbiology, University of Tokyo, for their kind help and criticism through out the investigation, and to Prof. T. Ogata for his helpful advice, especially on the immunological techniques. The author's thank should also go to Mr. S. Matsubara and Miss. H. Toda who kindly supplied purified PAB-TAA and M-PAB-TAA with α-phenylmaltoside.

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UREA DENATURATION OF TAKA-AMYLASE A

I. EFFECTS OF CALCIUM AND pH ON THE DENATURATION*

By SHIZUE FUJITA-IKEDA AND TOSHIZO ISEMURA

(*From the Faculty of Science and Institute for Protein Research, Osaka University, Osaka*)

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Previously we have measured the sedimentation, diffusion, viscosity and partial specific volume of native taka-amylase A and estimated its molecular weight and dimension, giving some considerations to the states of hydration and compactness of the molecule (*1*, *2*). It was concluded that taka-amylase A has a molecular weight of 51,000 and a molecular shape of a prolate ellipsoid with an axial ratio of about 4, and that the molecule is scarcely hydrated and considerably rigid under our experimental condition, *i. e.*, in 0.2 M acetate buffer at pH 4.8 with 0.01 M calcium acetate. It appears thus to be of importance to examine what is the nature of the force which would be responsible for maintaining native taka-amylase A molecule in such a state. One of the methods of approach to this problem is to break up the structure by rupturing the intramolecular bonds which maintain it. From this point of view, we have studied at first the urea denaturation of this protein.

According to Oikawa and Maeda (*3*), taka-amylase A crystallized from aqueous acetone solution in the presence of calcium acetate contains about 10 moles of calcium per mole of protein, among which 9 moles of calcium can be removed by dialysis against 0.02 M buffer (*e. g.*, acetate buffer at pH 5.6 and veronal buffer at pH 6.8 and 8.2, respectively) and the last 1 mole can be eliminated only by the dialysis in the presence of ethylenediamine tetraacetic acid. These authors observed that the enzymatic activity and optical rotation did not change during the process of removing the dialysable 9 moles of calcium but the elimination of the last 1 mole of calcium resulted in an increase of levo-rotation and loss of enzymatic activity. From these results they were lead to the conclusion that 1 mole of calcium is essential to maintain the active structure of taka-amylase A.

Since taka-amylase A is crystallized from aqueous acetone solution and stored in suspended state, the preparation of its solution for each use has need of dialysis to remove acetone. In our experiment it was found that the mode of urea denaturation is greatly influenced by the condition of dialysis even without ethylenediamine tetraacetic acid. As will be referred to later, the condition of dialysis allows to control the content of calcium bound to taka-amylase A. Even at a definite condition of dialysis the mode of urea denaturation varies necessarily with the pH of solution.

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In the present work, the effects of the dialysable calcium and pH on the urea denaturation was examined to find out the experimental condition favorable to the systematic investigations which will be described in the subsequent papers (*4*, *5*). The denaturation was investigated by means of the measurement of viscosity change of the solution. It was found that the viscosity of taka-amylase A in urea increases with time. Since the viscosity can be measured easily and is sensitive to the over-all molecular shape and compactness, its measurement offers a convenient means of studying the process of denaturation which changes with time.

EXPERIMENTAL

Taka-amylase A used in the present work was crystallized from "Taka-diastase Sankyo" by Akabori's method (*6*) and recrystallized three times from aqueous acetone solution containing 0.01 *M* calcium acetate. Special grade urea was recrystallized twice from 70 per cent ethanol (*7*).

The solutions of taka-amylase A with different calcium contents were prepared in the following ways: Taka-amylase A crystal suspended in aqueous acetone was centrifuged and then the precipitate was dissolved in water. [I] The solution was dialysed for more than 96 hours at 4° against the acetate or veronal buffer solution of which the ionic strength was about 0.1 and the pH was adjusted to a definite value required in each case. Under this condition taka-amylase A which would contain 1 molar equivalent of calcium is obtained (*3*). When a phosphate buffer was used, content of calcium was not determined. [II] The solution was dialysed for 67 hours at 4° against distilled water. Then 1 mole of this protein would contain some definite amount of calcium between 1 and 10 moles*. [III] After the solution was dialysed against distilled water, calcium acetate was added in such a way that the salt concentration would become 0.01 *M* when urea solution was added. In this case the protein with 10 moles of calcium would be obtained (*3*).

The procedure adopted to treat taka-amylase A with urea was as follows: Prior to mixing with each other, the protein solution prepared as mentioned above was diluted with a buffer solution or distilled water and the buffered urea solution was prepared in such a way that the concentrations of protein and urea, pH and ionic strength of the mixed protein-urea solution had required values. In most of the experiments the protein concentration of the mixed solution was about 0.5 per cent and the ionic strength was about 0.1. These two solutions were brought to the reaction temperature, mixed together and then the measurement of viscosity was started.

The concentration of taka-amylase A solution was determined by the micro-Kjeldahl method, assuming the nitrogen content of the protein to be 15.0 per cent (*6*). The concentration of urea solution was determined by simple weighing before dissolving. The concentrations of protein and urea in the protein-urea solution were calculated from the mixing proportion, ignoring the volume change on mixing.

The viscosity was measured by the viscometer of Ostwald type with the flow time of 120 to 180 seconds for water. On examining the rate of viscosity change during the denaturation the kinematic viscosity, $(\tau\text{-}\tau_0)/\tau_0 C$, instead of the reduced viscosity, was plotted against the reaction time, since the change in solution density with time can be traced accurately with great difficulty and is much smaller than that in flow time. Here τ is the flow time of solution (mixed solution of taka-amylase A with urea), τ_0 that of solvent (urea solution with the same composition as the mixed solution but without taka-amylase A) and C the protein concentration expressed in g. per 100 ml.

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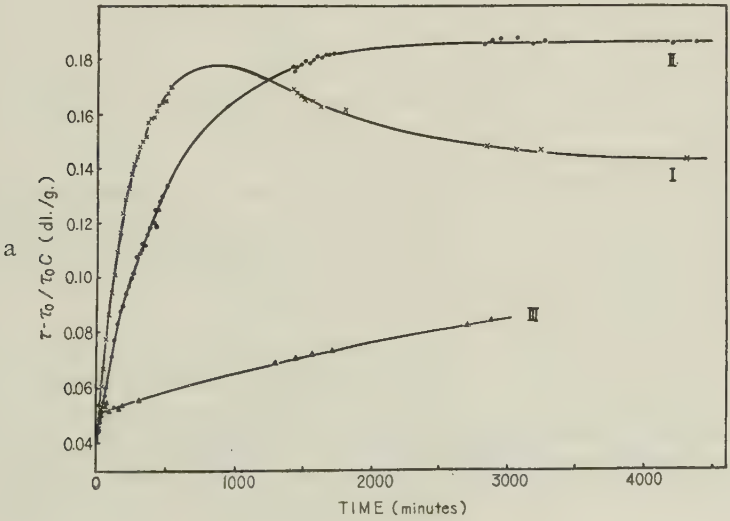
* Oikawa, A., personal communication

RESULTS

It was found that the rate of denaturation is affected markedly by the condition of dialysis even without ethylenediamine tetraacetic acid, *i. e.*, the amount of calcium bound to taka-amylase A. In Fig. 1 is shown the rate of denaturation of takaamylase A treated with 6 M urea at 20°, expressed by the increase in kinematic viscosity with time, for three different pH's. In all cases the kinematic viscosity immediately after the addition of urea was a value close to that of the native protein, and increased gradually or rapidly with time. At every pH, the rate of increase in viscosity was in the order, I>II>III, and consequently the lower the initial content of bound calcium, the faster the increase in viscosity.

As seen in Fig. 1b, the denaturation was faster in a phosphate buffer than in a veronal buffer, even at the same pH and in the same condition of dialysis. In curve II of the former case the calcium content was the same as in curve II of the latter case, before adding urea. Thus, it is likely that the higher affinity of calcium with phosphate than with veronal leads to the faster removal of calcium from the protein and, accordingly, to the faster denaturation of the protein by urea.



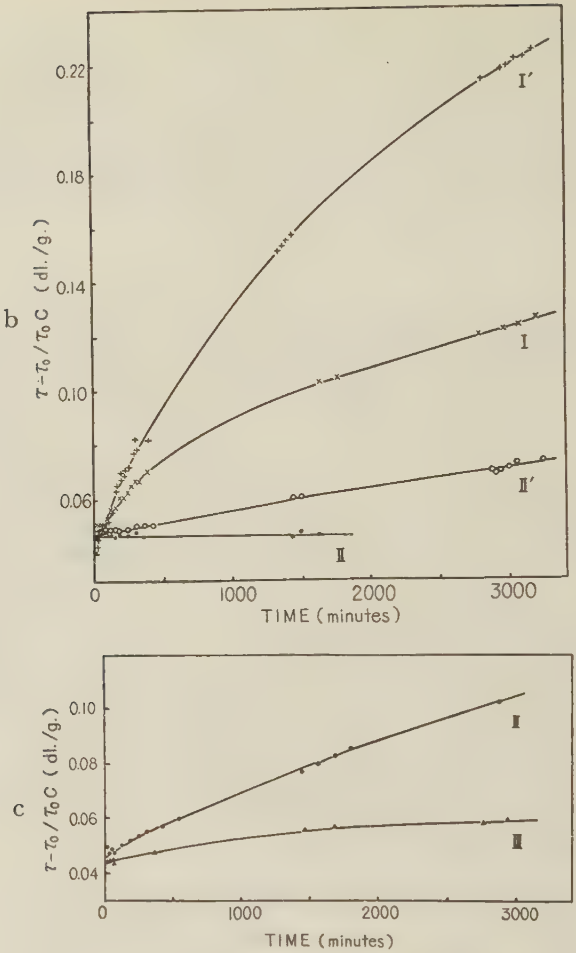


FIG. 1. Effect of the content of bound calcium on the kinematic viscosity of 0.5% taka-amylase A in 6 *M* urea at 20°. a, at pH 5.1 (0.1 Γ/2 acetate buffer); b, at pH 7.2 (I, II, 0.1 Γ/2 veronal buffer; I′, II′, 0.1 Γ/2 phosphate buffer); c, at pH 8.4 (0.1 Γ/2 veronal buffer).

Curves I(I′), II(II′) and (III) are runs for taka-amylase A solution of preparation [I], calcium content 1 mole (For I′ it was not determined whether or not exactly 1 mole of calcium was bound to the protein.), preparation [II], calcium content 1 to 10 moles, and preparation [III], calcium content 10 moles, respectively, as described in the experimental part.

Curve I in Fig. 1a passes a maximum followed by a gradual decrease. This indicates that the hydrolysis of the denatured taka-amylase A caused by a contaminant, taka-protease, takes place, as will be discussed later.

The effect of pH on the denaturation was also studied with taka-amylase A bound with the same amount of calcium. Fig. 2 shows the change in viscosity of taka-amylase A of preparation [II] in 6 *M* urea at 20°. It was

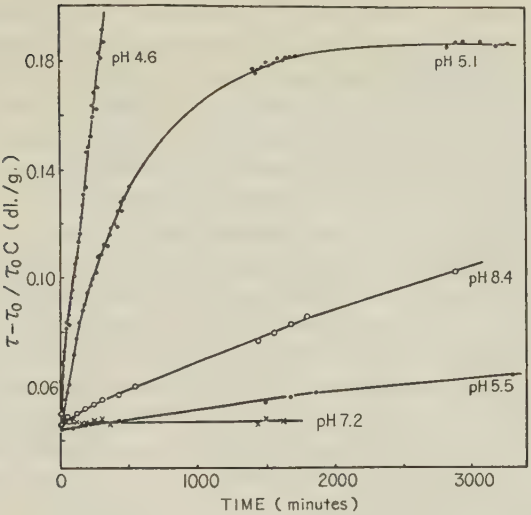


FIG. 2. Effect of pH on the kinematic viscosity of 0.5% taka-amylase A of preparation [II] (see the experimental part) in 6 *M* urea at 20°. pH 4.6–5.5, 0.1 Γ/2 acetate buffers; pH 7.2–8.4, 0.1 Γ/2 veronal buffers.

observed that pH 4.6 the viscosity increased without limit and the solution turned gradually turbid, finally giving a precipitate.

DISCUSSION

Role of Calcium in the Denaturation—From the present experimental results it may be concluded that the calcium, which is not essential for the maintenance of active structure of taka-amylase A and can be removed by the dialysis even in the absence of ethylenediamine tetraacetic acid, slows down the rate of viscosity increment of the protein in urea. In general, the viscosity increment of the protein solution indicates an unfolding of the relatively tightly folded native protein molecule to a more extended form. The increase in viscosity occurs also owing to the formation of a dissymmetric aggregate. However, the initial rate of viscosity increase shows the manner of unfolding, because it seems probable that the aggregation, even if it occurs, would be preceeded by the initial unfolding of the molecule. According to this view, the role of bound calcium is to prevent the polypeptide chain of taka-amylase A from the process of unfolding itself. Because of the experimental condition that the ionic strength was considerably high, *i. e.*, about 0.1, it seems unlikely that the protective action of calcium against urea denaturation can be attributed to the partial elimination of negative charges on ionized side chains causing the suppression of electric repulsion between them. Probably the formation of intramolecular calcium salt linkages would make the protein molecule resistant to urea denaturation. Along this line of reasoning it may be inferred that a calcium chelate structure stabilizes the tertiary and secondary structures of takaamylase A, the stability of which depends on the number of the intramole cular calcium salt linkages. It has been found (*3*) that the calcium atom which is dialysable only in the presence of ethylenediamine tetraacetic acid plays a different role from the other clacium atoms, in the maintenance of active structure of the protein molecule. This fact indicates that the stability of taka-amylase A is profoundly affected by the strength with which calcium is bound.

It has been reported (*8*) that taka-amylase A and *Bacillus subtilis* α-amylase is protected from the denaturation and inactivation to the same degree in the presence of calcium, suggesting that the protective effect of calcium on the inactivation is caused by the stabilization of the secondary intramolecular structure of these proteins. Fischer *et al.* (*9*, *10*) discussed the role of calcium in the molecular structure of all α-amylases so far investigated and suggested that the stability of various α-amylases towards proteolysis is determined by the number of metal ions bound to the enzyme and the strength with which these ions are retained. Our conclusion described above is in accord with these results.

Effect of Calcium on the Resistance towards Proteolytic Attack—It has been shown (*8*, *9*, *10*, *11*) that the calcium atom bound to α-amylase confers on the protein molecule a resistance to proteolytic attack. This fact was also confirmed in the urea denaturation of taka-amylase A. Curve I in Fig. 1a shows the existence of a maximum viscosity followed by a gradual decrease. It is known that α-amylases obtained from sources rich in proteases usually contain some proteolytic contaminants (*9*, *10*) and denatured taka-amylase A is hydrolysed by acid taka-protease (*12*). Curve I indicates, therefore, that taka-amylase A is first denatured by urea and then hydrolysed by taka-protease. (It is probable that taka-protease retains its activity even in such a concentrated urea solution as taka-amylase A is not allowed to remain native, since enzymes are generally stable in the presence of high concentration of substrates.) Curves II and III, for which the conditions other than the calcium content were identical with those for curve I, indicate no decrease in viscosity. This suggests that the proteolysis of taka-amylase A is inhibited when the amount of bound calcium is above a certain definite level. Conversely the above observation gives an evidence that taka-amylase A is contaminated with a trace of protease.

Separation of the Reaction of Denaturation from the Proteolytic Reaction—Curve I in Fig. Ia can be analysed by assuming the following reaction scheme:

$$\mathrm{N} \xrightarrow{k_1} \mathrm{S} \xrightarrow{k_2} \mathrm{H} \qquad (1)$$

where N represents the native taka-amylase A, S the urea-denatured one, H the proteolytic hydrolysates, and k_1 and k_2 represent the first order rate constants for the respective reaction. If the kinematic viscosities of N, S and H are expressed by η_N, η_S and η_H, and their concentrations by $[N]$, $[S]$ and $[H]$, respectively, it may be assumed that the observed value of kinematic viscosity at time t is given by

$$\eta=([N]\eta_N+[S]\eta_S+[H]\eta_H)/([N]+[S]+[H]) \qquad (2)$$

If $k_1>k_2$, the following relation should be fulfilled after a sufficient long time of reaction.

$$\eta-\eta_H=(\eta_S-\eta_H)\ e^{-k_2t} \qquad (3)$$

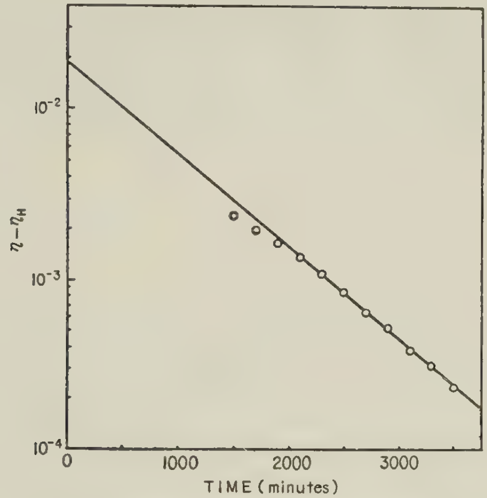


FIG. 3. Plot of log (η-η_H) against time for curve I in Fig. 1a.

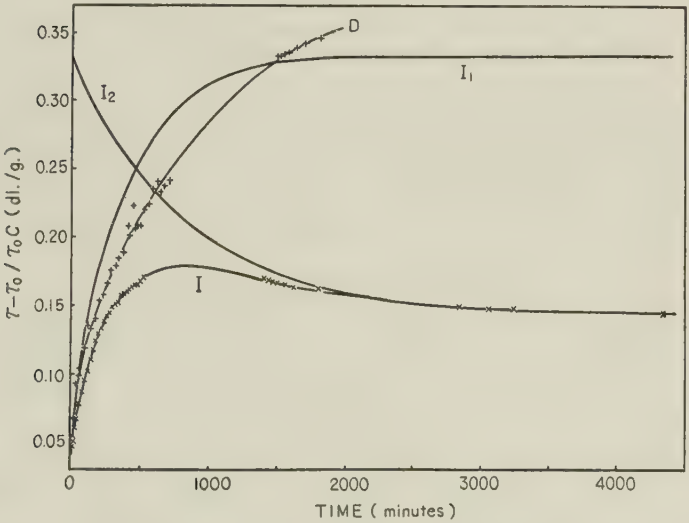


FIG. 4. Separation of the reaction of denaturation from the proteolytic reaction. Curve I, the same curve as curve I in Fig. 1a; curve I_1, the reaction of denaturation free from proteolytic degradation; curve I_2, the reaction of proteolytic degradation; curve D, the reaction of denaturation (under the same condition as for curve I) for taka-amylase A which had been recrystallized after treatment with diisopropylfluorophosphate.

By plotting log $(\eta-\eta_H)$ against t, a straight line is obtained, as shown in Fig. 3, where η_H is the final value of viscosity reached. The straight line has a slope and an intersection corresponding to $k_2=1.28\times10^{-3}$ min.$^{-1}$ and $\eta_S=0.333$, respectively. Using these values of parameters, Eq. (3) is plotted in Fig. 4 as curve I_2. Combining curve I_2 with the experimental curve I, we obtain curve I_1 which corresponds to the first reaction in scheme (1), *i.e.*, the reaction of denaturation free from proteolytic degradation. The plot of log $(\eta_S-\eta)$ against t where η is the viscosity value at t on curve I_1 but not on curve I, gives a stratight line with a slope equivalent to $k_1=2.49\times10^{-3}$ min.$^{-1}$, as shown in Fig. 5.

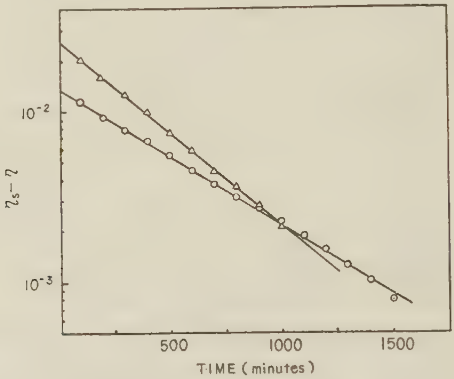


FIG. 5. Plots of log $(\eta_S\text{-}\eta)$ against time. ○, for curve II in Fig. 1a; △, for curve I_1, in Fig. 4.

It is known that some kinds of proteases, for instance, protease which contaminates hog pancreas amylase or *Bacillus subtilis* α-amylase, are inhibited by diisopropylfluorophosphate (*10*). It is expected, therefore, that this reagent would exert an inhibitory action upon taka-protease. Then taka-amylase A recrystallized after treatment with this reagent was examined under the same condition as that for curve I in Fig. 4. The viscosity-time curve is shown in Fig. 4 (curve D), which does not reveal any influence of proteolysis and may be compared with curve I_1, giving a relatively small discrepancy. The discrepancy in these two curves may be attributed to the configurational change of taka-amylase A itself caused by the treatment with diisopropylfluorophosphate.

Relation of the Rate and the Final State of Denaturation with the Content of Calcium—Curve II in Fig. Ia can be analysed by assuming the scheme

$$N \xrightarrow{k_1} S \qquad (4)$$

and the observed value of kinematic viscosity at time t to be

$$\eta=([N]\eta_N+[S]\eta_S)/([N]+[S]) \qquad (5)$$

The plot of log $(\eta_S-\eta)$ against t was found to be linear, giving $k_1=1.82\times10^{-3}$ min.$^{-1}$, as shown in Fig. 5.

The values of k_1 and η_S obtained by analysing curves I and II in Fig. Ia are summarized in Table I, together with the initial rates of viscosity increase in three curves, I_1 in Fig. 4 and II and III in Fig. la. It is found that the rate change in viscosity is faster and the final viscosity is greater, the lower the content of calcium. Modifications of the secondary or tertiary structure of the protein might involve the release of bound calcium. Therefore, the fact that the content of bound calcium before adding urea affects considerably the rate of denaturation appears to imply that the calcium atom is so strongly bound to the protein that it cannot be readily released by the structural change on denaturation. Furthermore, dependence of the final state of denatured protein on the initial content of calcium suggests that some of calcium remain still bound to the denatured protein.

TABLE 1

Effect of Content of Bound Calcium on the Denaturation

Preparation	Bound content of Ca moles/mole protein	k_1 *min.*$^{-1}$	η_S *dl./g.*	$(d\eta/dt)_t=0$ *min.*$^{-1}$
[I]	1	2.49×10^{-3}	0.333	14×10^{-4}
[II]	>1 and <10	1.82×10^{-3}	0.187	4×10^{-4}
[III]	10	—	—	0.3×10^{-4}

Effect of pH—The initial rates of increase in viscosity at various pH's can be estimated from Fig. 2 and are plotted against pH in Fig. 6. The measurements were made using two different kinds of buffers, though the ionic strengths were kept nearly constant at all pH regions. The result shown in Fig. 6 is of only semi-quantitative significance since buffer ions might have specific effects on the rate of denaturation. A part from this, Fig. 6 indicates

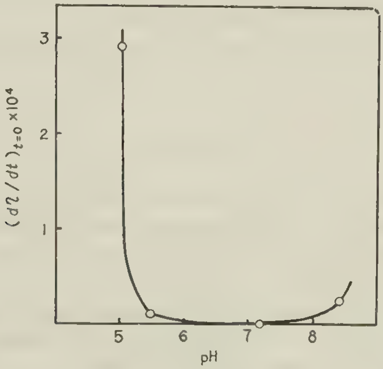


FIG. 6. Plot of the initial rate of viscosity increase against pH (estimated from Fig. 2.)

that taka-amylase A has its maximum stability towards urea denaturation near pH 6 to 7. This pH region does not coincide with the isoelectric point (3.5 to 4.0, at the ionic strength 0.1, 20°) or the isoionic point (3.80, at 20°).* Such a situation can be noticed in some of other proteins. For example, ovalbumin, the isoelectric point of which lies at pH 4.6 to 4.8, exhibits the minimum rate of urea denaturation at pH 7 to 9 (*13*). Accordingly, the effect of pH on the urea denaturation for these proteins cannot be explained simply by the electrostatic repulsion or the amount of net charge, which is minimum at the isoelectric region. This rather involves that some hydrogen bonds or salt linkages indispensable for the specific structure of protein are ruptured by hydrogen ions.

SUMMARY

1. The effects of calcium and pH on the urea denaturation of taka-amylase A were investigated by the measurement of solution viscosity. The viscosity of solution increased with time after the protein was mixed with urea, and, in some condition, reached a steady value.

2. The rate of denaturation was markedly affected by the conditions of dialysis, even without ethylenediamine tetraacetic acid, *i.e.*, by the amount of bound calcium which was not essential to maintain the active structure of the protein molecule. The increasing rate of viscosity was smaller as the content of bound calcium was higher. It seems that bound calcium play a role in stabilizing the secondary or tertiary structure of the molecule and inhibiting the molecular chain from unfolding itself.

3. When the amount of bound calcium was less than a certain definite one, taka-amylase A denatured in urea was hydrolysed by a trace of contaminant, taka-protease. This implies that the proteolytic degradation of taka-amylase A is inhibited by bound calcium.

4. Taka-amylase A had its maximum stability towards urea denaturation near pH 6 to 7.

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THE ASSOCIATION AND DISSOCIATION OF THE BACTERIAL α-AMYLASE MOLECULE

While determining the molecular weight of bacterial α-amylase from *Bacillus subtilis*, we found that sedimentation coefficient decreased on dilution. We found that the α-amylase of *B. subtilis* exists usually as a dimer which dissociates into two monomers at high dilution. However, recently Fischer *et al.* (*1*), showed that the sedimentation coefficient of α-amylase was not dependent on the concentration and that two α-amylase molecules might be held together by Zn^{2+} ion.

The bacterial α-amylase was supplied by Nagase Sangyo Co. Ltd. and had been prepared from the culture medium of *B. subtilis*. The enzyme was twice recrystallized by pH adjustment according to the method of Hagihara (*2*). The concentration of protein in the solution was estimated from the absorption at 280 mμ assuming that $E_{1cm}^{1\%}=25.6$. The sedimentation coefficient was measured in a Hitachi model UCA-1 type ultracentrifuge. Centrifugation was done at 55,430 and 60,000 r.p.m. at room temperature. Diffusion was studied by the schlieren cylindrical lens method using Neurath type cell.

The concentration of the amylase solutions used varied from 0.1 to 1.0 per cent. The $s_{20\,w}$ values calculated from the observed sedimentation coefficients were plotted against the protein concentration as shown in Fig. 1.

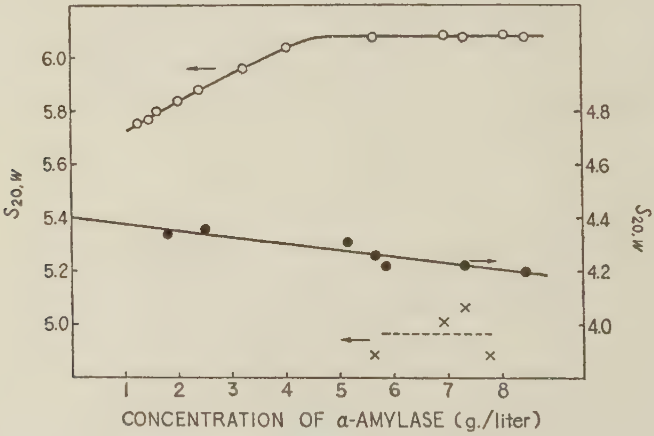

Fig. 1. The sedimentation coefficients, s_{20w}, of B. subtilis α-amylase under two conditions (○): pH 7.1, 0.005 M $Ca(CH_3COO)_2+0.1$ M NaCl; (●): pH 7.1, 0.005 M $Ca(CH_3COO)_2+0.01$ M Ca-EDTA+0.1 M NaCl; (×): sedimentation coefficient of slower moving component.

At a concentration of more than 8 g./liter, only a single sedimentation peak was observed with a constant sedimentation coefficient of $s_{20w}=6.10$ S. On the other hand, at a concentration of less than 5.5 g./liter there was a single peak deviating somewhat from the Gaussian curve and the s_{20w} decreased as a function of the protein concentration and the time of sedimentation. Between the above two concentrations, the sedimentation peak splits into two during the sedimentation as shown in Fig. 2 (a). The D_{20w} was calculated statistically to be 6.8×10^{-7} cm^2/sec by the inflection point method to be 6.0×10^{-7} cm^2/sec. This discrepancy in the calculation of the diffusion coefficient may be caused by the polydispersion of the amylase solution, one fraction with a sedimentation coefficient of $s_{20w}=6.08$ S and the other with lower s_{20w} of about 5.0 S.

Hitherto, a similar decrease in the sedimentation coefficient and splitting of the sedimentation peak on dilution of the protein has been reported for insulin (*3*) and α-chymotrypsin (*4*, *5*). In the case of α-chymotrypsin, Massey *et al.* explain this phenomenon by a monomer-dimer transformation.

According to Fischer *et al.*, α-amylase of *B. subtilis* is completely homogeneous as judged by electrophoresis and ultracentrifugation. Its sedimentation coefficient, s_{20w} was 6.47 S and its molecular weight was 96,900. On treating the amylase with Ca-EDTA (calcium salt of ethylenediamine tetra-acetic acid) the sedimentation coefficient decreased to 4.56 S and the molecular weight to 48,900. Accordingly, Fischer *et al.* concluded that two molecules of α-amylase of *B. subtilis* associate as a dimer bound by a Zn^{2+} ion. Removal of the Zn^{2+} ion by Ca-EDTA results in the liberation of two monomers.

In our investigation, the solution gave a single symmetrical peak corresponding to 4.40 S over the whole protein concentration range examined (0.2～1.0 per cent) after treating the amylase solution with Ca-EDTA as shown in Fig. 2, (b), and D_{20w} was 8.50×10^{-7} cm^2/sec. From these data the

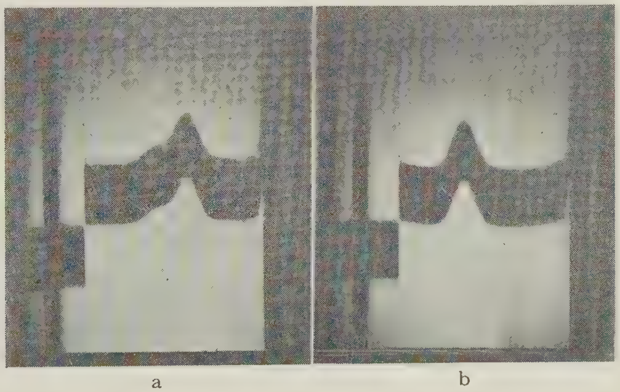

FIG. 2. The schlieren pattern of bacterial α-amylase in 0.005 *M* $Ca(CH_3COO)_2+0.1$ *M* NaCl solution.
(a): concentration of α-amylase 5.6 g./liter;
(b): concentration of α-amylase 5.4 g./liter treated with 0.01 *M* Ca-EDTA.

molecular weight seems to be 45,000.

The fact that only a single sedimentation peak was found at a concentration of less than 0.5 per cent, even before treatment of Ca-EDTA, may be due to the Johnston-Ogston effect or to a rapid equilibration of the monomer and dimer. In the light of the results obtained by Fischer *et al.*, our results suggest that the equilibrium occurs according to the following scheme:

$$2\ \text{Monomers} + \text{Zn}^{2+} \rightleftharpoons \text{Dimer}$$

This is probably reversed by dilution of the solution. Confirmatory evidence was also obtained by measurement of light scattering. Details of these experiments will be published shortly.

In conclusion, the authors would like to express their thanks to Nagase Sangyo Co. Ltd. for supplying the α-amylase and to Mr. Sugae who recrystallized some of it.

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Institute for Protein Research,
Osaka University,
Nakanoshima, Osaka.

TOSHIZO ISEMURA
KINJI KAKIUCHI
HACHIRO ETO*

(Received for publication, January 12, 1960)

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* On leave of absence from the Faculty of Science, Saga University.

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BIOSYNTHESIS OF SILK FIBROIN

I. INCORPORATION OF GLYCINE-C^{14} INTO PARTICLES OF POSTERIOR SILKGLAND IN VITRO

Previous works in this laboratory (*1*), (*2*) have shown that glycine-1-C^{14} was incorporated predominantly into protein of the posterior silkgland *in vivo* and large particles of this tissue incorporated the amino acid more actively than small one (microsome). In this paper an *in vitro* incorporation of glycine-C^{14} into the large particles is described. These particles were able to incorporate glycine-C^{14} in the absence of supernatant fluid or "pH 5 enzyme" of Hoagland *et al.* (*3*) from the posterior silkgland. This ability was almost completely destroyed by treating the particles with sodium deoxycholate (DOC). However, the ability was restored by an enzyme preparation obtained from the supernatant of the posterior silkgland. This enzyme fraction had little activity toward hydroxamic acid formation of glycine and glycine-dependent exchange of P^{32} or PP^{32} with ATP.

Preparation of Large Particles and Enzyme Fraction—The posterior silkgland of *Bombyx mori* was homogenized in a Waring blendor with four volumes of buffered medium containing 0.4 *M* of sucrose, 0.025 *M* of Tris-HCl (pH 8.0), 0.0025 *M* of KCl and 0.005 *M* $MgCl_2$. The homogenate was centrifuged once for 10 minutes at 700×*g.* to remove the unbroken cells. The large particles (RL) were then collected by centrifugation at 14,000×*g.* for 30 minutes at 0°. The supernatant fluid (SL) was brought to pH 6.1 with 0.1 *M* acetic acid, and the precipitate (E_1) was centrifuged at 8,500×*g.* for 8 minutes and then the second supernatant fluid was acidified to pH 4.9. The resulting precipitate (E_2) contained most of the activity for stimulation of incorporation of glycine-C^{14} into the particles as described below. The final supernatant fluid was denoted as E_3.

Preparation of DOC-treated Particulate Fraction (*RD*)—RL was treated with two per cent DOC solution for a few minutes at 0°, diluted with four volumes of the buffered 0.4 *M* sucrose solution described above and immediately centrifuged at 14,000×*g.* for 15 minutes. The sediment (RD) was resuspended in the same medium and centrifuged at 14,000×*g.* for 20 minutes.

Measurement of Glycine Incorporation—The reaction systems consisted of 10 μmoles of ATP, 5 μmoles of phosphoenolpyruvate, 0.04 mg. of pyruvate kinase, 0.07 μmoles glycine-1-C^{14} (300,000 c.p.m.), 5 μmoles of $MgCl_2$, 50 μmoles of Tris-HCl (pH 8.0), 50 μmoles of KCl, 400 μmoles of sucrose, an amino acid mixture containing 19 amino acids except glycine, 0.2 ml. of RL or RD suspension (3 to 4 mg. of protein) and 0.2 ml. enzyme solution (1.5 mg. of protein) in a final volume of 1 ml. These systems were incubated at 37° for 30 minutes. After the incubation, the protein was precipitated with 1 ml.

of 20 per cent $HClO_4$ containing 15 per cent glycine, washed twice with 6 per cent $HClO_4$, boiled with 6 per cent $HClO_4$ for 10 minutes and then centrifuged. It was further washed once with 5:1 ethanol: 6 per cent $HClO_4$, warmed with 3:1 ethanol: ether at 50° for 60 minutes and its radioactivity was counted in a windowless gas-flow counter.

Assay Procedure of Glycine Activation—Hydroxamic acid formation and P^{32} or PP^{32} exchange reaction were performed using a system similar to that of Hoagland *et al.* (*3*) and DeMoss *et al.* (*4*).

As shown in Table I, active incorporation of glycine-C^{14} was observed when RL was incubated alone. In this system the supernatant fractions, SL, E_1, E_2, E_3 revealed no stimulating effect for the incorporation of glycine-C^{14} into the protein of RL. On the other hand, the extent of the incorporation of glycine-C^{14} into RD (DOC-treated particles) was very low when it was incubated alone, but markedly stimulated by the addition of the supernatant fraction, E_2 (Table II).

It is of interest to point out that RD recombined with E_2 is as active as RL and also that GTP is effective in this system, agreeing with the results of Keller *et al.* (*5*). However, the enzyme in the E_2 fraction is considerably different from the "pH 5 enzyme" of Hoagland *et al.* in enzymatic activity.

TABLE I

Incorporation of Glycine-C^{14} into Protein of Cellular Fractions of Posterior Silkgland

Incorporation system¹⁾	Radioactivity c.p.m. per mg. of protein
SL²⁾	9
RL³⁾	156
RL plus SL	173
RL plus E_1 ⁴⁾	164
RL plus E_2 ⁴⁾	155
RL plus $E_2(0)$ ⁵⁾	1
RL plus E_3 ⁴⁾	108

1) All systems contained 0.2 μ moles of GTP
2) Supernatant fluid after removal of large particles
3) Large particles
4) Enzyme fractions from SL
5) Incubation time was zero.

As shown in Table III, E_2 had little enzymic activity in catalyzing the formation of glycine hydroxamate and also glycine-dependent exchange of P^{32} or PP^{32} with ATP, although these enzymic activities were quite active in both the initial supernatant fluid (SL) and the fraction E_3. Soluble RNA prepared by Kirby's method did not replace this enzyme. Recently Ochoa *et al.* (*6*) reported an enzyme, "amino acid incorporation enzyme", which stimulated the incorporation of amino acids into protein of *Alcaligenes* *faecalis* particles. It is not clear at this time whether our enzyme from the silkgland is of the same nature as Ochoa's enzyme. It would, however, appear that the enzyme from the silkgland is more directly concerned with some phase of biosynthesis of silk fibroin that "pH 5 enzyme" of Hoagland *et al.* The purification and characterization of the enzyme from the silkgland are now under investigation,

TABLE II

Effect of Fraction E_2 on Incorporation of Glycine-C^{14} into Protein of Posterior Silkgland

Incorporation system	Radioactivity c.p.m. per mg. of protein	
	exp. 1	exp. 2
RL	119	
RD[1)]	9	7
E_1		10
E_2	11	
RD plus E_1		28
RD plus E_2	159	126
RD plus E_2 plus GTP[2)]	286	
RD plus E_3		7

1) DOC-treated particulate fraction
2) 0.2 μ moles of GTP

TABLE III

Hydroxamic Acid Formation and P^{32} or PP^{32}-ATP Exchange

Fraction	Hydroxamic acid formed	PP^{32} exchanged	P^{32} exchanged
SL	0.4	0.57	0.0
E_1	0.0	0.0	0.0
E_2	0.0~0.15	0.0~0.02	0.0
E_3	0.5	0.99	0.0
RL	0.05	0.03	0.0

The data are presented as micromoles per hour per mg. of protein

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Laboratory of Biochemistry
Department of Agricultural Chemistry
Tohoku University, Sendai

IWAO SUZUKA
KENSUKE SHIMURA

(Received for publication, January 12, 1960)

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BIOSYNTHESIS OF SILK FIBROIN

II. INCORPORATION OF SPECIFIC C^{14}-LABELED AMINO ACIDS INTO PROTEIN OF POSTERIOR SILKGLAND IN VITRO

During the course of an investigation on the incorporation of radioactive glycine, alanine and leucine into large particles of the posterior silkgland o *Bombyx mori*, it was observed that a specific particulate fraction and a less specific enzyme are involved in the incorporation of each amino acid.

Cellular fractions (intact large particles (RL) and deoxycholate(DOC)-treated particulate fraction(RD)) and enzyme (E) were prepared from the posterior silkgland of *Bombyx mori* and *Attacus ricini* respectively by the method described previously (*1*). Each fraction is denoted as BRL, BRD and BE for *Bombyx mori*, and as TRD and TE for *Attacus ricini*, respectively, as shown in Table I and II. Microsomes (RM) and pH 5 enzyme (RH) of the rat liver were prepared by a method similar to that of Hoagland *et al.* (*2*). The reaction mixture for incorporation contained: approximately 4 mg. of protein

TABLE I

Incorporation of Specific C^{14}-labeled Amino Acids into Particulate Proteins

Incorporation system	C^{14}-labeled amino acid	Radioactivity c.p.m. per mg. of protein		
		exp. 1	exp. 2	exp. 3
BRL[1]	glycine			128
BRL	alanine			61
BRD[2] plus BE[3]	glycine	65	126	92
BRD plus BE	alanine	30		
BRD plus BE	leucine	12		
RM[4] plus RE[5]	glycine	7	5	
RM plus RE	leucine	22	27	
BRD plus RE	glycine	55		41
BRD plus RE	leucine	9		4
RM plus BE	glycine	15	22	
RM plus BE	leucine	35	73	

1) Intact large particles of the posterior silkgland of *Bombyx mori.*
2) DOC-treated particulate fraction of the posterior silkgand of *Bombyx mori.*
3) Enzyme fraction of the posterior silkgland of *Bombyx mori.*
4) Microsomes of the rat liver.
5) pH 5 enzyme of the rat liver.

of RL or RD plus 1.5 mg. of protein of E; 10 μ moles of ATP; 5 μ moles of phosphoenolpyruvate; 5 μ moles of $MgCl_2$; 50 μ moles of KCl; 50 μ moles of Tris-HCl; 400 μ moles of sucrose; 0.04 mg. of pyruvate kinase; 0.2 μ moles of GTP and either glycine-1-C^{14} (300,000 c.p.m. per 0.07 μ moles) or DL-alanine-1-C^{14} (600,000 c.p.m. per 0.14 μ moles) or DL-leucine-1-C^{14} (600,000 c.p.m. per 0.14 μ moles). The mixture was incubated for 30 minutes at 37°. After the incubation, the protein was precipitated with six per cent $HClO_4$, washed, dried and counted in a windowless gas-flow counter.

As shown in Table I, among of three amino acids tested glycine was most actively incorporated into the particulate proteins in the system of BRL alone or BRD plus BE of the posterior silkgland *Bombyx mori*. For alanine the incorporation activity was reduced to about one half of that for glycine, and for leucine it was about one fifth of that for glycine. It is of interest that this order of the activity corresponds rather well with the proportion of these amino acids in the silk fibroin (*Bombyx mori*). In contrast with this, the system (RM plus RE) of the rat liver, in which leucine is contained in about three times of glycine, incorporated C^{14}-leucine more active than glycine. Recently Schweet *et al.* (*3*) observed that the ratios of the amount of C^{14}-labeled leucine, isoleucine and valine incorporated into the rabbit hemoglobin coincided closely with the proportions of these amino acids in the globin. When the DOC-treated particulate fraction of *Bombyx mori* (BRD) was recombined with the enzyme from the rat liver (RE) instead of *Bombyx mori* enzyme (BE), incorporation of glycine was more predominant than that of leucine. In the recombined system with BE plus liver microsomes (RM) instead of BRD, on the other hand, incorporation of leucine was greater than that of glycine.

TABLE II

Incorporation of Specific C^{14}-labeled Amino Acids in Cell-free Systems of Posterior Silkgland of Attacus ricini

Incorporation system	C^{14}-labeled amino acid	Radioactivity c.p.m. per mg. of protein
RM plus TE[1)]	leucine	48
RM plus TE	alanine	30
TR[2)] plus RE	leucine	24
TRD plus RE	alanine	148

1) Enzyme fraction of the posterior silkgland of *Attacus ricini*

2) DOC-treated particulate fraction of the posterior silkgland of *Attacus ricini*

Similar results, as shown in Table II, were obtained in the experiments using the particulate fraction of *Attacus ricini*, of which silk fibroin contains about 40 per cent of alanine, 28 per cent of glycine and one per cent of leucine. In the system with TRD plus RE incorporation of alanine was more predominant than that of C^{14}-leucine, and in the system with RM plus TE leucine was incorporated more actively than alanine.

These results provide evidence that incorporation of specific amino acids is under the control of the DOC-treated particulate fraction as the acceptor for the amino acids. Thus it is probable that the large particulate fraction acts as one of functional factors governing the amino acid sequence of the silk fibroin, and that the enzyme which stimulates incorporation of amino acid into the particulate fraction or the pH 5 enzyme of Hoagland *et al.* may be less important than the particulate fraction or microsomes in controlling the specificity of protein being synthesized.

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Laboratory of Biochemistry
Department of Agricultural Chemistry
Tohoku University, Sendai

IWAO SUZUKA
KENSUKE SHIMURA

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